UNIVERSITY OF TASMANIA

Demonstration and Manipulation of Acquired Resistance to Amoebic Gill Disease in Atlantic Salmon, *Salmo salar L.*

By

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Submitted in fulfilment of the requirements for the

Degree of Doctor of Philosophy

University of Tasmania (October, 2000)

Declaration of Originality

I declare, to the best of my knowledge, that the material contained within this thesis is original except where due acknowledgment is given. It has not been accepted for the award of any other degree or diploma.

Vanessa L Findlay
October, 2000

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ABSTRACT

Amoebic gill disease (AGD), caused by *Paramoeba* sp., is the most serious infectious disease infecting sea caged Atlantic salmon, *Salmo salar* L., in Tasmania, Australia. After AGD had been described from Tasmanian salmonids, it also emerged as a problem in other countries. Major outbreaks have occurred in France, Spain and Ireland and disease, although not to the same extent, has been reported in the US and New Zealand.

In Tasmania at present, treatment consists of a series of freshwater baths given during the critical high temperature, high salinity periods of the year. These baths last between two to three hours and during a normal summer fish are usually treated two to three times. However, there have been summers when up to eight baths have been undertaken and it appears recently, that this scenario is becoming more common.

Because of the time and monetary costs associated with AGD, the Tasmanian industry is constrained with regards to increasing production and thus it's competitiveness in international and domestic markets. Any strategy that would eliminate or even reduce the number of baths required would be of substantial commercial value. All attempts to find a practical chemotherapeutic agent have been fruitless, thus immunisation and/or immunostimulation appeared to offer the best hope of providing an alternative treatment strategy.

This project reports for the first time, the presence of varying degrees of resistance to AGD in fish that have been previously infected and then treated using freshwater bathing and demonstrates that this resistance can be modulated via the use of the immunostimulant, levamisole. It is also the first report of the use and efficacy of levamisole as an immunostimulant of the nonspecific defence system of Atlantic salmon.

In order to achieve the results obtained a number of novel and improved experimental techniques were developed and are reported here.

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INTRODUCTION

Amoebic gill disease (AGD), caused by *Paramoeba* sp., is the most serious infectious disease affecting and constraining the production of sea-caged Atlantic salmon, *Salmo salar* L. and rainbow trout, *Oncorhynchus mykiss* (Walbaum) mariculture in Tasmania (Munday, Foster, Roubal & Lester, 1990). Mortalities of up to 2% per day, reaching a total of 50% have been recorded (Munday *et al.*, 1990). Indeed, this disease, together with the suboptimal osmoregulatory performance of rainbow trout in full salinity seawater, has led to this species being cultured almost exclusively in brackish water in Tasmania. Prior to the spring of 1984, when significant quantities of Atlantic salmon and rainbow trout were first placed in sea cages at various sites in Tasmania, the disease had been previously undescribed in Australia (Munday, 1985).

The Tasmanian Atlantic salmon industry generates more than $100 million (Smithies[1] *pers. comm.*, 1998) annually and is one of the most important industries in the State of Tasmania. It is envisaged the industry will expand production to $400 million by the year 2010. Threats to the expansion of the Australian industry include the improving Norwegian and Chilean products, the introduction of imported salmonid product to the domestic market and the changes required in husbandry techniques to cope with more intensive culture. Strategies in place to deal with these threats include production cost minimisation programmes, marketing campaigns to promote home-grown product and the conduct of cutting edge research and development. At present, however, the greatest threat to the Tasmanian salmonid industry comes from AGD.

In France, AGD has emerged as a major problem in sea-farmed Atlantic salmon, but it is of lesser importance in rainbow and brown trout, *Salmo trutta* L. (Baudin Laurencin[2], *pers. comm.*, 1995). Major outbreaks of AGD have been described in Atlantic salmon farmed in Ireland (Rodger & McArdle, 1996; Palmer, Carson, Ruttledge, Drinan & Wagner, 1997) and the disease has been reported in this species in Chile (Groman[3], *pers. comm.*, 2000) and Washington State (Findlay, Zilberg & Munday, 2000). Recent outbreaks of AGD in Atlantic salmon in Spain have been extremely serious and it has been suggested that these may result in the collapse of

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[2] Baudin Laurencin, F. CNEVA, Brest, France
[3] Groman, D. Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island, Canada

the industry (Echabe[4], *pers. comm.*, 2000). Outbreaks in the Pacific salmons, have been minor and sporadic, and it may be that these species are inherently resistant to the disease. Kent, Sawyer & Hedrick (1988) reported minor out-breaks in coho salmon, *Oncorhynchus kisutch* (Walbaum), in Washington State and California, and C. Anderson (*pers. comm.*, 1995) has diagnosed occasional outbreaks in chinook salmon, *Oncorhychus tshawytscha* (Walbaum), held in poor water conditions in New Zealand. There have also been reports of AGD due to a *Paramoeba* sp. occurring in turbot (*Scophthalmus maximus* L.) in Spain (Dykova, Figueras, Novoa & Casel, 1998)

In Australia, prior to the introduction of out-of-season smolts, infestations of *Paramoeba* sp. associated with clinical disease were only seen when water temperatures rose above 12°C. However, with the introduction of out-of-season smolts clinical disease may occur in these fish even when temperatures drop below 12°C. It appears that salinity has a more marked effect with clinical disease occurring only when salinities approach 35‰. This was demonstrated in the summer of 1995 when a major field study failed due to a large freshwater spike that maintained the fish in salinities of less than 8‰ for more than a week. Clinical disease was not experienced on that particular site at all during that summer. While this is the situation in Tasmania, in Washington State and Spain strains from some species are able to stand significantly lower salinities. Kent *et al.*, (1988) reported that the optimal salinity for growth of the Washington State strains was between 15 and 20‰. Dykova *et al.*, (1998) and Paniagua, Fernandez, Ortega, Parama, Sanmartin & Leiro (1998) grew the strains isolated from turbot at 22‰ and 10-30‰, respectively. Growth at these salinities suggests that these strains are euryhaline.

At present, amoebic gill disease of salmonids in Tasmania is controlled by a series of freshwater baths given during the critical high temperature, high salinity periods of the year (ie usually November to April/May). These freshwater baths last between two and three hours and during a normal summer fish are usually treated two to three times. However, there have been summers when up to eight baths have been undertaken and it appears recently that this scenario is becoming more common. Freshwater bathing adds significant cost to the production of Tasmanian salmonids. Any strategy that would eliminate or even reduce the number of baths required would be of substantial commercial value. All attempts to find a practical chemotherapeutic agent have been fruitless thus immunization and/or immunostimulation appear to offer the best hope of providing an alternative treatment strategy.

[4] Echabe, A. Luso-Hispana de Acuicultura, Puerto de Meiras, Spain

The most outstanding feature of amoebic gill disease is the presence of excessive mucus on the gills. Microscopically, the most significant pathology is hyperplasia of the epithelia leading to fusion of the secondary lamellae and the formation of large interlameller vesicles or cysts (Kent *et al.*, 1988; Munday *et al.*, 1990). Ultramicroscopically, the pseudopodia of the amoeba can be seen penetrating cavities at the surface of the degenerating epithelial cells or even invading cell junctions (Roubal, Lester & Foster, 1989).

While *P. pemaquidensis* is regarded as the cause of amoebic gill disease of salmonids in Australia (Roubal *et al.*, 1989; Munday *et al.*, 1990), it is also widely described as a free-living organism (Page, 1973). Although this amoeba is readily grown on artificial media, the cultured organisms have proven to be non-pathogenic to salmonids (Kent *et al.*, 1988; Howard & Carson, 1993). Amoebic gill disease can, however, be produced in naïve salmonids by cohabitation with diseased fish (Findlay *et al.*, 1995), or by exposure to ameobae isolated from the gills of fish with AGD (Zilberg & Munday, 2000). This suggests that virulence factors crucial to the establishment of disease are absent from the cultured Paramoeba organisms.

There is a considerable body of anecdotal evidence suggesting that fish that have been previously exposed to AGD become relatively resistant to re-infection (Percival[5] *pers. comm.*, 1995). However, studies using cultured and wild harvested *Paramoeba* sp as immunogens both parenterally and enterically or as polyclonal antisera against *Paramoeba* have failed to provide protection against the disease (Akhlaghi *et al.*, 1996; Zilberg & Munday, 2000a). This anomalous situation was one of the facets investigated in this study.

Clarification of the nomenclature used within this study is needed since confusion may surround the identification of the parasite that causes amoebic gill disease. Using immunoperoxidase staining, Howard and Carson (1993) have shown that their isolate is the predominant organism on the gill of diseased salmonids. However, they have not speciated the organism. Kent *et al.*, (1988) identified the organism causing gill disease in Washington State as *Paramoeba pemaquidensis* and by using complete 18S rDNA sequences (2104 bp) for 4 Tasmanian and one each Irish and USA *P. pemiquidensis* strains associated with AGD, Wong & Elliot (2000) have confirmed that the Tasmanian *Paramoeba* strains were identical and the Irish and USA strains had >98% similarity. However, the fact that Washington State and

[5] Percival, S. Aquaculture Development and Veterinary Services Pty Ltd, Allens Rivulet, Tasmania

Spanish isolates can grow at 10-30‰, which is significantly lower than that tolerated by the Tasmanian isolates, suggests that the *Paramoeba* organisms causing AGD consist of a number of subspecies, or possibly even species. As a result, I have used *Paramoeba* sp. when referring to the causative organism of AGD.

The initial aim of this study was to scientifically test the hypothesis that salmonids develop relative resistance to reinfection with *Parameoba* sp. after one or more attacks of AGD. In order to achieve this, a reproducible system for producing AGD in susceptible species has been developed and techniques for quantifying disease and immune parameters optimized.

CHAPTER 1

LITERATURE REVIEW

Contents

The purpose of this review is to provide an outline of the fish immune and defence response so as to more adequately demonstrate interactions between Atlantic salmon and Paramoeba sp.. The review is not intended to be a comprehensive examination of the immune and defence capabilities of fish.

DESCRIPTION OF THE DEFENCE SYSTEMS IN FISH

Two types of immune responses occur in vertebrates including fish: the specific, that combines humoral and cellular components directed against specific antigens and the nonspecific component that depends principally on phagocytic cells (cellular component) and soluble antimicrobial factors (humoral component) (Anderson, 1974; Ingram, 1980; Fletcher, 1982). Immunity, however, is a relative term and there are fine lines of distinction between the terms resistance, immunity and non-susceptibility.

Resistance

Resistance usually describes some barrier that prevents the entry into and/or attachment to an otherwise susceptible host of a pathogen. These barriers may be physical or chemical. In fish, the skin, gills and alimentary tract usually provide the first line of defence. All of the above surfaces are covered with mucus that not only forms a physical barrier but may also contain factors that inhibit the penetration and survival of a pathogen. Studies on resistance to, rather than immunity against, pathogens by fish are sparse. However, there is some evidence (Bowers & Alexander, 1981) demonstrating that fish that are treated with an hyperosmotic solution prior to exposure to a microorganism become more susceptible to invasion. These workers demonstrated that when brown trout (*Salmo trutta*) were exposed to hyperosmotic stress, *Escherichia coli* could be isolated from the blood stream soon after. In contrast no bacteria could be isolated from non-stressed fish. This suggests that the disruption to the gill epithelium caused by hyperosmotic stress renders the normally resistant fish at risk of invasion. Thus, it may be concluded that the physical barrier provided by the epithelium and their mucus secretions plays an integral, but sometimes over-looked, role in protection against infection and preservation of internal homeostasis.

While chemical defence may form a significant part of resistance under this definition, it is more appropriately dealt with for the purposes of this thesis under the heading of nonspecific defence. Once the physical barriers have been breached, the host must rely on cellular and humoral defences and if there has been no prior exposure to the pathogen then these defences would be nonspecific (Fletcher, 1982).

Non-specific defence system

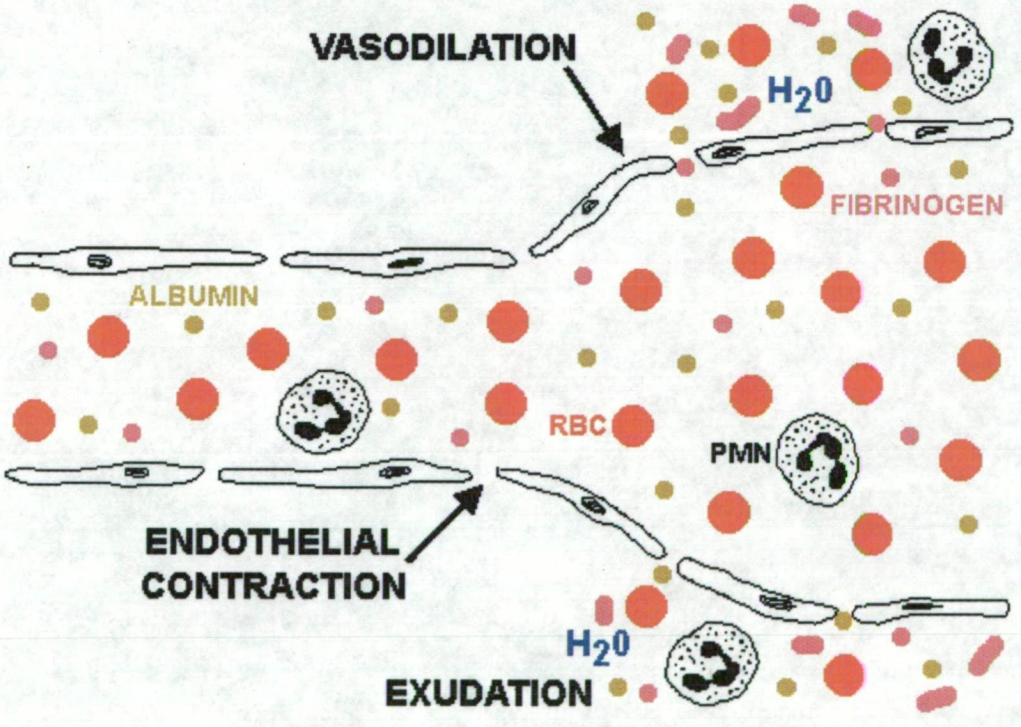
The first and often most important responses of fish to infectious agents are non-specific (Blazer, 1991). This is particularly true of cool and cold water fish because the development of the specific immune response is temperature dependent (Avtalion, 1981), with antigen specific responses occurring more slowly at lower temperatures. The intrinsic broad-spectrum barriers of the non-specific defence response important in fish include the inflammatory response, various microbial inhibitory substances of the mucus and sera, and the circulating and tissue phagocytes (Ellis, 1982a; Anderson, 1992; Raa, 1996). In fact the nonspecific natural substances found in normal healthy fish such as precipitins, lysins, agglutinins and C-reactive protein may help to overcome various diseases before the specific immune responses occur (Dash, Saha, Sahu & Gangal, 1993).

The Inflammatory Response

The basic response of all vertebrates to tissue injury is inflammation (Kreier & Mortensen, 1990). The rupture of the basement membrane, in tissues where these occur, provides a site for the interaction of the pathogenic agent with the cells and humoral factors able to limit the spread and rid the area of injurious agents and damaged tissue. This sets the stage for healing. Three major events occur during the inflammation process (Figure 1):

- An increased blood supply to the area
- Increased capillary permeability that allows the soluble mediators of immunity to reach the site of infection
- The migration of monocytes (in particular monocyte derived macrophages), neutrophils and cytotoxic cells towards the area

Figure 1 The inflammatory response



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Non-specific cellular defence

Although many soluble factors, serum proteins and cells participate in the inflammatory response, the phagocytic cell and its products maintain the central role.

Macrophages have been recognized as the principal phagocytic cell and play a vital role in many aspects of piscine immune responses. Although they do not have immune specificity (Clem, Sizemore, Fellsaesser & Miller, 1985), macrophages are important accessory cells in initiating specific immune responses and are also potent effector cells capable of killing a wide range of pathogens (Secombes, 1990). Many studies have demonstrated macrophages to be highly phagocytic for inert and antigenic material (Ellis, Munro & Roberts, 1976; McKinney, Smith, Haines & Sigel, 1977; Braun-Nesje, Bertheussen, Kaplan & Seljelid, 1981).

It is a widespread belief that two important differences exist between macrophages of some teleost species and those of mammals. The first is the apparent lack of Fc and C3 receptors on fish macrophages and the second, the lack of opsonic activity of fish antibody and complement (Wrathmell & Parrish, 1980). These differences suggest that the mechanisms of phagocytosis and intracellular killing, both of which are enhanced by the antibody-complement complexes in mammals, may be different in some fish species (Ellis, 1982a).

There are results, however, that indicate that salmon macrophages possess receptors that bind to human complement factors, C3b and C3bi, and agarose beads coated with these factors are readily phagocytosed (Johnson & Smith, 1984). Furthermore, Matsuyama, Yano, Nakao & Yamakawa (1991) reported carp C3 to be involved in phagocytosis by carp peritoneal neutrophils. Elevated phagocytosis was displayed when sheep red blood cell and yeast were treated with normal carp serum. Also treatment of the neutrophil surface protein with trypsin significantly decreased phagocytosis, suggesting the presence of C3 receptors. Similarly, Nonaka, Iwaki, Nakai, Nozaki, Kaidoh, Nonaka, Natsuume-Sakai & Takahashi (1984) demonstrated that antisera to trout C3 inhibited the phagocytosis of complement-coated sensitized erythrocytes.

Historically, the role of neutrophils in fish has been confused and poorly defined (Ellis, 1982a). Numerous authors suggested that there was very little evidence that neutrophils in fish were actively phagocytic, in fact, they have been reported to have no phagocytic properties (Ellis *et al.*, 1976; McKinney *et al.*, 1977) or to be, at best, weakly phagocytic (Young & Chapman, 1978). However, it is now well established that fish neutrophils are phagocytic (MacArthur & Fletcher, 1985) with the occurrence of a biphasic (ie neutrophils followed by macrophages) inflammatory response. Further, the findings that fish neutrophils possess most of the enzymes found in mammalian neutrophils suggest they play an active role in defence mechanisms.

It has been suggested that the fish neutrophil may carry out a bactericidal role extracellularly rather than intracellularly (Ellis, 1982a). Neutrophils can kill by both oxygen dependent and independent mechanisms, including nitric oxide. They produce a more intense respiratory burst than macrophages and their secretory granules contain highly cytotoxic proteins such as acid hydrolases, myeloperoxidase, lactoferrin and lysozyme (Roitt, Brostoff & Male, 1993).

While it is well recognized that there is an increase in the number of eosinophilic granulocytes (Lester & Daniels, 1976; Reimschusessel, Bennett, May & Lipsky, 1987), neutrophils and macrophages (Sommerville, 1981; Pilsford & Matthews, 1984; Roubal, Lester & Foster, 1989) after a fish succumbs to infection the role that each of these cells plays in the immune and defence response is still perplexing.

Non specific humoral defence

The serum and mucus of fish contains a number of substances, which are not immunoglobulins, that affect the growth of micro-organisms (Alexander, 1985). Alexander (1985) classified these substances into the following four groups according to the affect they have on the invading organism: (a) microbial growth inhibitory substances; (b) enzyme inhibitors; (c) lysins; and (d) agglutinins and precipitins.

Microbial growth inhibitory substances

These substances act either by depriving the invading organisms of essential nutrients or by interrupting metabolic pathways. Many of the acute phase proteins act to inhibit growth of an invading parasite and are so-called because the concentration of a number of these inhibitory substances increases rapidly during an infection. Transferrin displays anti-microbial properties when not fully saturated, and consequently has been shown to play a role in the pathology of many infections by limiting the amount of endogenous iron presented to pathogens, by chelating the metal, and making it unavailable for use by the organisms (Weinberg, 1974). The role of transferrin in disease resistance in fish has been examined using a number of species. The results, however, are contradictory and there seems to be a lack of standardization between and within experiments. Winter, Schreck & McIntyre (1980) demonstrated that fish with effective variants of transferrin showed low mortality when infected with BKD, *Renibacterium salmoninarum*. Conversely, Withler & Evelyn (1990) concluded that the differences in resistance to BKD they demonstrated were not related to the effectiveness of the transferrin type, as fish which showed the greatest resistance had only 4% TfC and the least resistant strain had 27% TfC. It is, however, agreed that certain genotypic variants are better able to

compete for iron than others and resistance lies in the differential avidities of the different transferrin types for iron binding (Ingram 1980). Winter *et al.*, (1980) concluded that whether or not genotypically effective transferrin confers a disease resistance advantage may be irrelevant in commercial species as the fish exhibiting effective transferrin grew so much slower than those with a less effective variant. Thus, it is unlikely that this type of resistance is being selected for in salmonid populations.

Caeruloplasmin is another acute phase protein. It complexes with divalent metal cations such as copper but its defence role would appear to be more through its ability to oxidise ferrous iron to ferric, thus increasing the removal of iron from the environment, than as a chelator of copper (Alexander & Ingram, 1992). Modulation of caeruloplasmin concentration in fish may occur as a result of contact with certain substances. Syed, Coombs & Keir (1979) demonstrated that fish that had been exposed to the aquatic metal pollutant cadmium displayed elevated concentrations of caeruloplasmin.

Interferon is perhaps the most important of the proteins that interferes with the replication and growth of potential pathogens. The defensive mechanism allows infected cells some measure of protection and the viral-inhibiting protein either modifies or prevents the development of lesions (Baron, Brunell & Grossberg, 1979). It has been shown in salmonids that interferon has a specific protective affect upon cells and an inhibitory affect on virus replication (DeKinkelin & Dorson, 1973; Okamoto, Shirakura, Nagakura & Sano, 1983). Interferon may also play a significant role in the activation of macrophages. Graham & Secombes (1988, 1990) demonstrated that rainbow trout leukocytes produced macrophage activation factor (MAF) after mitogen stimulation and that the MAF from these leukocytes also conferred resistance to infectious pancreatic necrosis virus. It is probable that this MAF is in fact a Type II interferon.

Enzyme Inhibitors

While it recognized that the antiproteinases present in the serum of vertebrates primarily function to maintain homeostasis of the blood and other body fluids they also act to inhibit the actions of the many parasites that carry out extra-organism digestion of the host tissue.

Many of the proteinase inhibitors of mammals have not been identified or isolated from fish (Alexander & Ingram 1992). However, the "all-purpose" proteinase inhibitor, α2-macroglobulin, which inhibits enzymes from all four of the proteinase groups, has been reported to occur in fish by Ellis, Hastings & Munro (1981).

α2-macroglobulin principally inactivates pathogens via a "wrap-round" method, however it can also bind covalently to some enzymes and adhere to others (Alexander & Ingram 1992). There seems to be contrasting views on the action of proteinase inhibitors in the process of disease resistance. Ellis *et al.* (1981) and Ellis (1987) demonstrated that the proteases of *Aeromonas salmonicida* were inhibited by the α - globulins in fish serum while Ellis & Stapleton (1988) concluded that the susceptibility of salmonids to furunculosis correlated directly with the activation of the proteases of *A. salmonicida* by components of the fish serum.

Fish serum also contains other common groups of protease inhibitors such as inhibitors of serine proteinases, cysteine proteinases, aspartic proteinases and metallo proteinases (Raa, 1996).

Substances which cause lysis

These substances are all enzymes that act either singularly such as lysozyme and proteinase or in a complex such as that observed in the complement system. These enzymes act to degrade or digest components of an attacking organism.

Lysozyme

Lysozymes are ubiquitous enzymes (Jolles & Jolles, 1984). In fish, lysozyme has been detected in the blood, mucus and many other tissues (Fletcher & White, 1973; Lindsay, 1986; Grinde, Lie, Poppe & Salte, 1988). There is a wide variation in the concentration of lysozyme detected in different teleost species. The salmonid family and in particular rainbow trout have high levels of lysozyme when compared to members of the cod and tuskfish families (Grinde *et al.*, 1988).

The specific substrate of lysozyme is β-(1-4) linked *N*-acetyl-*D*-glucosamine, N-acetyl-muramic acid peptidoglycan. Lysozyme appears to be involved in defence against viruses, neoplasms, bacteria, and possibly fungi and insects.

There seems to be some inconsistencies in the demonstration of the presence and levels of lysozyme. Although there are numerous methods to measure lysozyme described in the literature, only two are used consistently:- turbidimetric method (Parry, Chandan, & Shahani, 1965) modified by Sankaran & Gurnani (1972) and the lysoplate assay (Osserman & Lawlor, 1966). Both of these assays rely on the ability of lysozyme to lyse cells of *Micrococcus lysodeikticus* and it appears that optimal results depend on a range of variables including the pH and ionic strength of buffers. There has not yet been an international standardization of the methods thus it seems that lysozyme concentration and activity reported by different authors cannot be

directly compared. In some cases even experimental results that have been obtained using identical methods cannot be compared because they are expressed in different units.

Other non-specific lysins

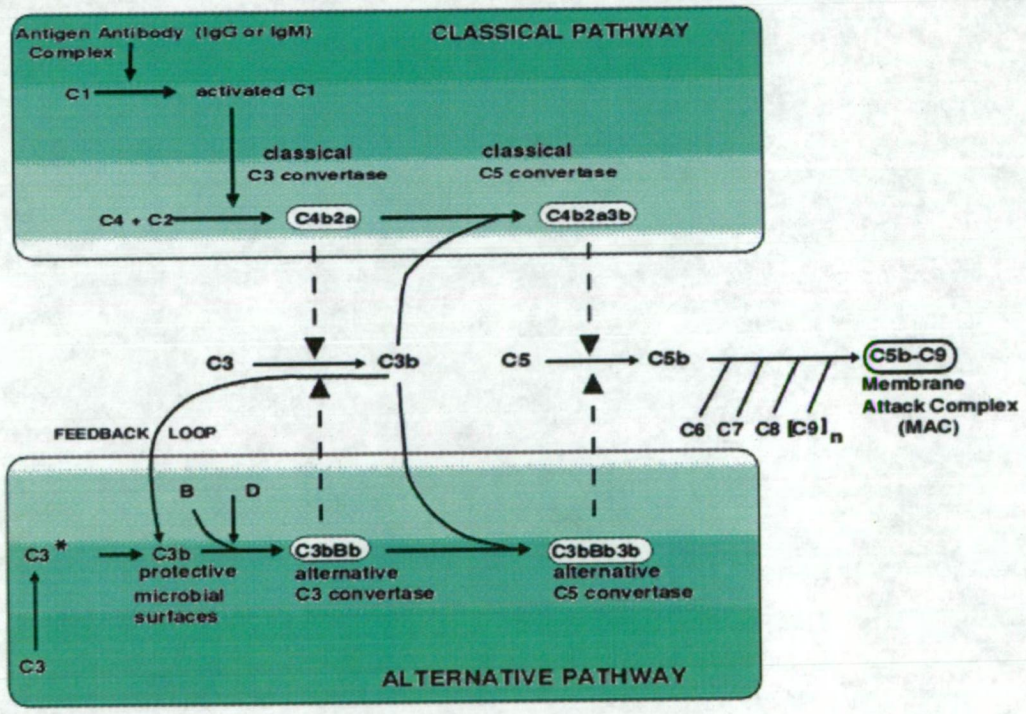
Besides lysozyme, a variety of lysins have been described from the mucus and serum of fish. Hjelmeland, Christie & Raa (1983), demonstrated that the skin mucus of the rainbow trout, *Oncorhynchus mykiss*; charr, *Salvelinus alpinus*; cod, *Gadus morhua*; coalfish, *Gadus virens*; redfish, *Sebastes marinus*; and plaice, *Pleuronectes platessa* contain proteolytic enzymes which appeared to be identical to the pylorus caecal trypsin, but is secreted into the mucus. Itami (1993) described the bacteriolytic activity of the skin mucus of ayu, *Plecoglossus altivelis* and demonstrated the presence of antibody, lysozyme, haemagglutinin and a vibriolytic substance. While several authors have demonstrated the presence of chitinase and chitobiase in various organs and blood of teleosts (Okutani & Kimata, 1964; Fletcher & White, 1973; Fange, Lundblad & Lind, 1976; Goodrich & Morita, 1977) it appears that their roles and derivation is, as yet, undetermined. A number of authors have claimed that these enzymes may have a protective function against invasion by chitin-containing fungi and invertebrate parasites (Fange *et al.*, 1976; Ingram, 1980). However Lindsay (1986) demonstrated that the pattern of enzyme distribution was not consistent with a role in defence. Furthermore, Fletcher & White (1973) showed that the presence of chitinase was closely related to the diet of the fish and that its activity was lost from non-feeding fish.

Complement

The complement system is a complex composed of at least 18, and possibly 20 proteins, of which 11 have been isolated and characterized (Porter, 1977). The most recognized role of complement is its' ability to affect cell membranes leading to osmotic lysis and cell death. Further consequences of complement activation include increased vascular permeability, chemotaxis of leukocytes and their retention at sites of tissue injury, and the enhancement of phagocytosis (Fletcher, 1982).

The complement cascade can be activated by two different, but convergent, pathways: the classical and the alternative. The immunoglobulin-dependent, or classical pathway is comprised of three operationally defined functional units: the recognition unit (C1): the activation unit (C2, C3 and C4) and the membrane attack mechanism (C5, C6, C7, C8 and C9) (Ingram, 1980). The alternative pathway of complement is activated by a wide variety of substances including polysaccharides and lipopolysaccharides, derived from the cell walls of fungi and bacteria and the exoskeletons of animals, such as crustacea and insects (Sakai, 1999); bacterial endotoxins (Sakai, 1992); and animal venom factors (Day, Gewurz, Johannsen, Finstad & Good, 1970). This alternative mechanism by-passes complement components C1, C2, and C4; originates via C3 and leads from C5 up to C9 (Ingram, 1980). The activation of the alternative pathway is free from antigen-antibody complexes; and is therefore nonspecific (Sakai, 1992) (Figure 2).

Figure 2 Complement pathways



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Both the classical and alternative complement pathways have been demonstrated in fish. Nonaka, Yamaguchi, Natsuume-Sakai & Takahashi (1981) demonstrated the lysis of rabbit erythrocytes with LPS-treated trout serum in the absence of antibody. This suggests that an alternative pathway of complement activation exists in trout. Similarly, Engstad, Robertsen & Frivold (1992) reported an increase in haemolytic

activity in glucan-treated fish. Sakai (1981, 1983), Jenkins & Ourth (1991) and Watts (2000) have also reported immunoglobulin-independent haemolysis, indicative of an alternative pathway, in carp, rainbow trout, catfish and tuna respectively. Recently, Halvor & Robertsen (1997) detected the complement component C3 in the mucus of β-glucan treated Atlantic salmon.

Several authors have stated that the alternative pathway is of primary importance in the lower vertebrates. Roberts (1978) suggests that the importance of the properdin pathway resides in the possibility that antibody is not required to initiate the complement reaction, thus it has a great advantage in the poikilotherms, where conditions are not always favourable for the protein synthesis necessary for production of antibodies. Kaastrup (cited in Alexander, 1985) even goes so far as to say that the alternative pathway is the only one present in fish. However, it has been demonstrated in numerous fish species that IgM has the ability to fix complement and activate its lytic properties. Cushing (1945) demonstrated that carp serum contained three of the four classic components found in guinea pig serum. Nonaka, Iwaki, Nakai, Nozaki, Kaidoh, Nonaka, Natsuume-Sakai & Takahashi (1984) have similarly shown the complement system in rainbow trout has many similarities to mammalian complement. The presence of complement or haemolytic activity has also been recorded from the albacore, *Thunnus alalunga* (Giclas, Morrison, Curry, Laurs & Ulevitch, 1981), brown trout (Ingram, 1987) and bluegill, *Lepomis macrohirus* (Smith, Potter & Mechant, 1967). Nevertheless no fish species has been found that contains all of the components C1 to C9 recognized in the mammalian classical pathway (Sakai, 1992).

C-reactive protein

CRP is a member of the acute phase protein group and while its function remains uncertain it has been reported to be involved in phagocytic activity, opsonisation, interactions with complement, anti-bacterial lysis and agglutination (Pepys, 1981; Alexander, 1985). 'CRP-like' proteins are widely distributed among both invertebrates and vertebrates (Kreier & Mortensen, 1990) and fish are no exception. CRP has reported in many teleosts including the plaice, *Pleuronectes platessa*; eel, *Anguilla japonica*; lumpsucker, *Cyclopterus lumpus*; rainbow trout, *Onchorhynchus mykiss*; and tilapia, *Sarotheroden mossambicus* (Alexander & Ingram, 1992). In fish, unlike the situation in mammals, CRP is present at most times and at levels up to 500 times greater.

C-reactive protein resembles immunoglobulins in several properties and its broad specificity for group-specific C-polysaccharides (mucopeptide and *N*-acetyl galactosamine-6-phosphate) commonly found in the cell walls or surface structures

of invading organisms suggests it may afford a nonspecific primary defence role (Fletcher, 1982).

Agglutinins and Precipitins

Essentially, precipitation and agglutination is simply the aggregation of molecules and cells respectively. Like other vertebrates, fish possess a variety of substances that cause the agglutination and/or precipitation of carbohydrate residues. These factors are physico-chemically, biologically and antigenically distinct from antigen-stimulated, immune antibodies or immunoglobulins (Alexander & Ingram, 1992). Even so, there is some confusion in the literature between non-Ig agglutinins and natural Igs that has led to the term antibody being used in reference to both the above.

Goldstein & Poretz (1986) defined a lectin as "a carbohydrate-binding protein (or glycoprotein) of non-immune origin which agglutinates cells and/or precipitates glycoconjugates" and now, nonspecific agglutinins and precipitins are generally recognized under the term lectin. These agglutinins have been identified in fish. However studies on the specificities and functions of natural haemagglutinins (lectins) in fish are sparse.

Immunity

It was stated earlier in this review that there is a fine line of distinction between resistance, immunity (both passive and active) and non-susceptibility. Perhaps the distinction between nonspecificity and specificity could identify appropriate lines. In a general sense, a distinction between innate and acquired could also represent an appropriate distinction; with innate aligning with nonspecific and acquired with specific.

The word 'immune' is derived from the Latin word 'immunis', which means 'safe by exemption', and therefore strictly speaking, would include all means by which an organism may rid itself of invading pathogens. However, nonspecificity may be more appropriately aligned with resistance and innate natural defence, while the terms immunity, immunise and immune response tend to reflect an acquired selective response or the induction of a selective response, in which specific antibodies or a specific attack sequence is produced against invading organisms. Immunity is generally attributed to an inherited ability to produce antibodies, whether this is natural immunity where antibodies are produced against certain pathogens without actual stimulation by homologous antigens from the pathogens, such as described in the section on agglutinins and precipitins, or acquired immunity where a host is

stimulated to produce antibodies by contact with antigens. Where this line becomes less clear is with the phenomenon of passive immunity, where antibodies produced by one animal are transferred to another to engender immunity. While the donor species must still be able to produce antibodies, the recipient of these antibodies need not be immunocompetent. Strictly speaking, for the individual involved, this may fall outside the above definition of immunity.

The second component of specific immunity, cell-mediated immunity, is not directly associated with killing via antibodies but, *inter alia*, initiates their production. Many of the wide ranging aspects of this immune response are intrinsically linked with the nonspecific defence system.

Semantics aside, for the purpose of the next part of this literature review, immunity will be discussed in the terms of specific responses both cell mediated and humoral.

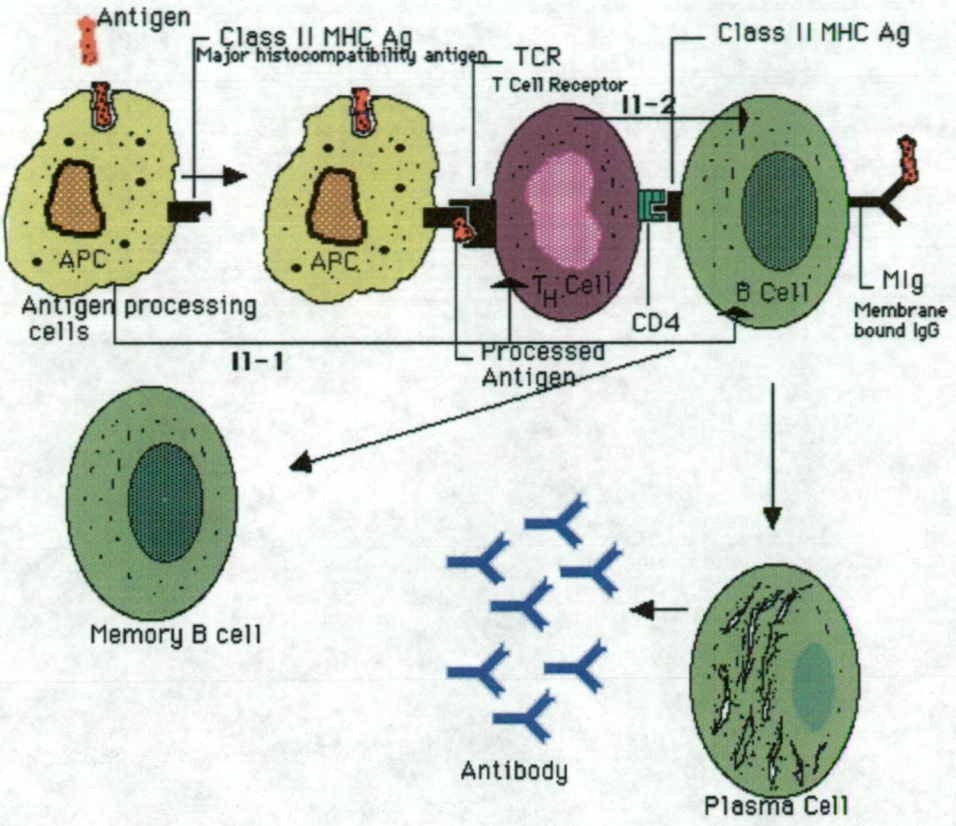
Specific Immune System

Specific Humoral Immunity

Humoral immunity relies on the circulation of antibodies within the fluid system of a host to combat pathogens. Antibodies belong to the group called immunoglobulins and the striking feature in teleosts is the presence of only one class of immunoglobulin (Ig) (compared to five in mammals) (Ellis, 1982a). This immunoglobulin is most like mammalian IgM and can be found in most of the tissue fluids in fish including plasma, lymph, skin, gut and gill mucus and bile (van Muiswinkel, 1995). More recently, there has been some evidence that a second class of immunoglobulin may be present in fish. Wilson, Bengten, Miller, Clem, Dupasquier & Warr (1997) reported the presence of immunoglobulin, much like human IgD in channel catfish (*Ictalurus punctatus*).

Like mammals and birds, fish have two main populations of lymphocytes, T lymphocytes and B lymphocytes. Although the source of these cells in fish in unknown it is likely to be the kidney (Ellis, 1988). On exposure to an antigen the T and B cells cooperate in the response but it is the B cells that form the major component of specific humoral immunity. Once the antigen has been presented to the B cells, these differentiate into plasma cells which produce antibody specific to the stimulating antigen or into cells which are capable of becoming plasma cells on subsequent exposure to antigen and are therefore called memory cells (Ellis, 1988). Figure 3 illustrates the events leading to the production of antibodies.

Figure 3 Events leading to the production of antibodies



Reproduced after Perez (2000)

Specific Cell Mediated Immunity

Specific cell mediated immunity is T cell dependent and refers to all the manifestations of the specific immune response which do not involve antibodies (Gudkovs, 1988). Although T helper cells are necessarily involved in the production of antibodies and therefore remain linked to the humoral immune response, the T cell population possesses clones that are responsible for cell-mediated immunity (Rijkers, 1982).

The T lymphocyte population can be divided into distinct groups according to the function of that sub-population. According to Ellis (1988) and Kennedy-Stoskopf (1993), these include:

- Cytotoxic killer cells that are capable of lysing foreign cells
- Lymphokine producing cells which produce substances that enhance the killing capacity of macrophages through activation
- Suppressor cells which regulate the production of antibodies and lymphokines

In general, the cell-mediated component of the specific immune response includes the hypersensitivity reactions, transplantation responses, inhibition of macrophage migration and the mixed lymphocyte reaction. To these, can be added the helper function as mentioned above. Certain aspects of cell mediated immunity are not necessarily manifest to the benefit of the animal and there have been a number of studies done which demonstrate these affects of cellular immune reactions and the kinetics of this component.

Taking evidence provided in studies on transplantation and graft reactions in fish (see reviews by Cushing, 1970; Ellis, 1982b; Secombes, 1991; Stet & Egberts, 1991), essentially, the cellular reactions in fish are the same as for mammals. Rijkers (1982) summarised the cellular immunological characteristics of fish that are in accordance with mammals:

- Second-set grafts are rejected more rapidly than first-set
- Immunological memory is long-lived; memory is donor specific
- Xenografts (from different species) are rejected faster than allografts (from the same species)
- No isohaemagglutinating antibodies are involved in the rejection process
- The antigen dose does not affect the median survival time of the grafts
- Only living tissue evokes immunological memory

Hypersensitivity reactions have not been thoroughly studied in fish, though there is evidence that they do exist. It is thought that 'Strawberry disease' (of unknown aetiology) in salmonids, may be related to allergic or hypersensitivity reactions (Kfoury, Okamoto, LaPatra, Tanaka, Suzuki, Motonishi & Ikeda, 1994). Similarly, hypersensitivity has been shown to be active in other granulomatous diseases of fish, such as tuberculosis and lymphocystis with sensitized lymphocytes demonstrated in the later stages of disease (Timur, Roberts & McQueen, 1977a,b). Baldo & Fletcher (1975) reported hypersensitivity skin reactions in various flatfish species in reaction to C-polysaccharide substances and Bartos & Sommer (1981) found that rainbow trout developed a hypersensitivity reaction against *Mycobacterium tuberculosis* and *M. salmoniphilum* in the skin and the thymus.

Hypersensitivity reactions often coincide with the development of macrophage migration inhibition that, as the name suggests, inhibits the movement of macrophages. In animals that have been sensitized with an appropriate antigen, a lymphokine that inhibits movement of macrophages in the surrounding area is released from T-like lymphocytes. Macrophage migration inhibition has been shown to occur in both elasmobranchs and teleosts (Gudkovs, 1988). In particular, Smith, McCarthy & Paterson (1980) showed that the lymphocytes of the brown trout, *Salmo*

trutta fario, immunized against *Aeromonas salmonicida* inhibited macrophage migration in the presence of antigen.

The aspect of cell-mediated immune responses in fish against pathogens is little explored (van Muiswinkel, 1995). However, together with the studies mentioned above, the following workers have demonstrated the cellular immune function in teleosts. Thomas & Woo (1990) and Li & Woo (1995) demonstrated a cell-mediated immune response in rainbow trout. There was evidence of antibody independent cell-mediated cytotoxicity in fish vaccinated against *Cryptobia salmositica*. Whyte, Chappell & Secombes (1989) showed that *in vitro* killing of the digenean cercariae, *Diplostomum spathecum* was not increased using antiserum-coated larvae or *in vivo*-activated macrophages individually, but when they were combined increased killing occurred.

IMMUNOSTIMULATION

By definition, an immunostimulant is a chemical or drug that elevates nonspecific defence mechanisms or the specific immune response (Anderson, 1992). In the strict sense of the word, immunostimulation has been used for close to a century with reports from the early 1900s on the use of lanolin, paraffin and other oils in conjunction with vaccines to increase antibody production and vaccine efficacy (McKercher, 1986). Because the processes of the specific immune and nonspecific defence systems are interwoven and highly interdependent, as previously discussed, when an immunostimulant enhances the nonspecific defence system it will also activate processes in the specific immune system (Raa, 1996). For this reason, I firmly believe that although a large proportion of the studies on immunomostimulation in fish report only on the specific immune component, the substances that were used should be considered for their potential to modulate the nonspecific defence system. At this point in time there is no reason to believe that the application of immunostimulants in most circumstances is not modulating both the nonspecific and specific components of a fishes response.

Sindermann (1984) recognized that the immune system will be influenced by the genetic constitution, age, sex, nutritional status and hormonal balance of the fish, with the additional impact of environmental variables, both natural and man-induced. In the intensive aquaculture situation whereby some of the above factors may be compromised, aquatic animals become highly susceptible to infectious disease. During the last decade, immunostimulants have become recognized as potential substances for heightening the activity of the defence mechanisms in aquatic animals and conferring protection against disease.

Recognized immunostimulants in fish, include bacterial cell wall fragments, β-1,3-glucans of yeast and mycelial fungi, peptides and a number of synthetic products (Raa, 1996). There are many functions that may be modulated when an immunostimulant is used to treat fish. However, the most widely recognised and the most often demonstrated is that of phagocytic regulation. Given the important role of phagocytes in the uptake of antigens, this too adds weight to the postulate that in most circumstances both the specific and nonspecific functions are being modulated.

There have been three excellent reviews on immunomodulation in fish in recent times by Anderson (1992), Raa (1996) and Sakai (1999), so there is no need to go over common ground in this review.

A summary of the immunomodulators that have been used in fish and the affects these had on various nonspecific defence system and specific immune functions is presented in Table 1 (Appendix 1). This table has been put together from information in the reviews mentioned above as well as an extensive literature review.

GILL-ASSOCIATED AMOEBAE INFECTIONS IN FISH

Gill associated ameobae have been reported in finfishes since the early 1900s. Until recently most reports of these amoebae were from finfish reared in freshwater (Kubota & Kamata, 1971; Sawyer, Hnath & Conrad, 1974; Sawyer, Hoffman, Hnath & Conrad, 1975; Taylor, 1977; Daoust & Ferguson, 1985; Nash, Nash & Schlotfeldt, 1988). These amoebae were placed in various genera including *Thecamoeba*, *Amoeba*, *Acanthamoeba*, *Naegleria* and *Vahlkampfia*. However, during the late 1980s, and until present, there have been an increasing number of marine fishes from an increasing number of locations affected by gill-amoeba. All verified pathogenic marine amoebae have so far, been from the genus *Paramoeba*.

***Paramoeba* spp.**

Amoebae of the genus *Paramoeba* Schaudinn, 1896, are exclusively marine and have a wide distribution throughout the world. Amoebae belonging to the genus *Paramoeba* are characterized by the presence of a parasome in close relationship to the nucleus (Cann & Page, 1982; Page, 1973). Despite the consistency of the structural characteristic, the various species within this genus show great ecological variability.

Many of the species described from this genus are free living, including the type species *P. eilhardi*, *P. schaudinni*, and *P. aesturina* (Cann & Page, 1982). Little else is known about them, other than *P. aesturina* can be cultured on artifical media (Cann & Page, 1982). *P. perniciosa* is regarded as an obligate parasite of the blue crab (*Callinectes sapidus*), and has not been grown on artificial media (Sprague, Beckett & Sawyer, 1969). *P. invadens* is a significant pathogen of sea urchins (*Strongylocentrotus droebachiensis*), can be cultured on artificial media and disease can be produced in susceptible hosts using cultured organisms (Jellet & Scheibling, 1988). Recently, significant mortality occurred in lobsters in Long Island, California due to a *Paramoeba* species (Cawthorn[4], *pers. comm.* 2000). Investigations on this outbreak are continuing.

Munday (1986) reported the occurrence of disease associated with a marine amoeba in farmed salmonids early in 1985 but at that stage the organism had not been identified. It was not until 1988 that Kent *et al.*, (1988) and Munday, Foster, Roubal & Lester (1990) described severe disease associated with *Paramoeba* in salmonids reared in seawater in California and Washington and Tasmania, respectively. These were the first reports of *Paramoeba* affecting a vertebrate. Subsequent *Paramoeba* infections in a range of fish species including, Atlantic salmon, coho salmon, rainbow trout, brown trout and turbot have been reported in Ireland (Rodger & McArdle 1996; Palmer, Carson, Ruttledge, Drinan & Wagner, 1997), Chile (Groman *pers. Comm.*, 2000), France (Baudin Laurencin *pers. comm.*, cited in Findlay & Munday, 1998), Spain (Paniagua, Fernandez, Ortega, Parama, Sanmartin & Leiro 1998; Dykova, Figueras, Novoa & Casel, 1998; Echabe *pers. comm.*, 2000.) and New Zealand (Anderson[5] *pers. comm.*, 1990).

Of the *Paramoeba* species that infect aquatic animals, it is the one found in seawater reared finfish that has been of greatest interest to aquaculturists and fish health scientists. It has been tentatively speciated as *P. pemaquidensis*. While this species has been implicated as the cause of disease in numerous species of the salmonid family the reasons why this normally free-living organism colonizes the gills is still not completely understood (Nowak & Munday, 1994). It is interesting to note that the reports of many of the freshwater amoeba include accounts of bacterial infection in association with the amoebae. These workers concluded that the amoebic infection may have been opportunistic as a result bacterial growth; the overgrowth of bacteria provided a rich source of nutrition for the normally free-living amoeba. Roubal, Lester & Foster (1989) did not find large bacterial loads in association with *Paramoeba* infections in marine fish although Garland (*pers. comm.* cited in

[4] Cawthorn, R. Atlantic Veterinary College, University of Prince Edward Island, Prince Edward Island, Canada

[5] Anderson, C. Ministry of Agriculture Fisheries, Upper Hutt, Wellington, New Zealand

Munday, Lange, Foster, Lester & Handlinger, 1993) found increased bacterial loads by culture.

Structure of *Paramoeba pemaquidensis*

Paramoeba pemaquidensis may appear in two forms. Amoebae removed from the gills of salmonids commonly exhibit many fine radiate pseudopodia (Plate 1). This transitional or floating form is 20 to 30 μm in diameter (Cann & Page 1982; Munday *et al.,* 1990). The locomotive form is elongated with blunt pseudopodia (Plate 2). They measure between 20 and 35 μm along the longest axis (Cann & Page 1982).

Ultrastructurally, and depending on the plane of section, a nucleus and one or two parasomes may be seen. At times, dense condensations of chromatin occurs at the poles of the parasome (Munday *et al.*, 1990).

Plate 1 Transitional or floating form *Paramoeba* sp.

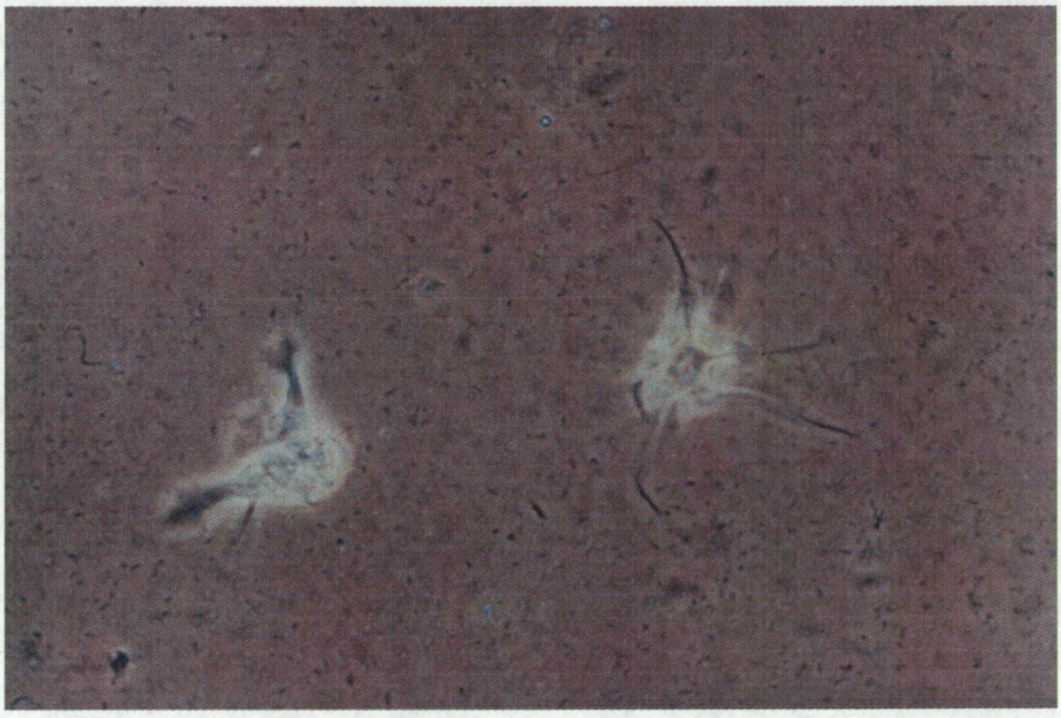
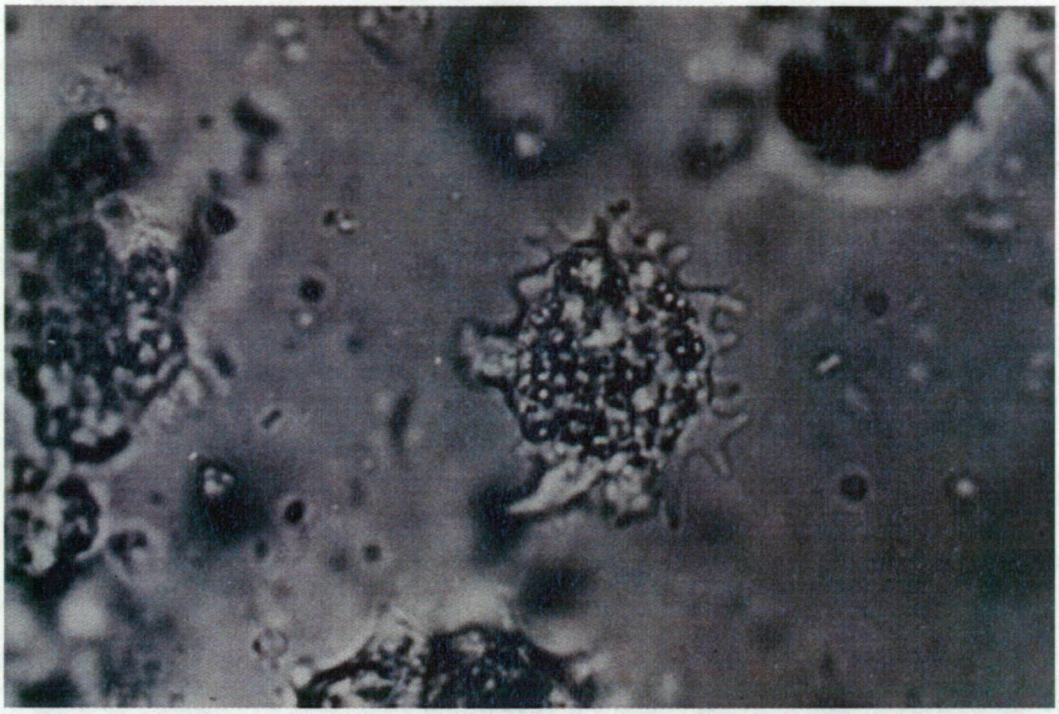


Plate 2 Locomotive form *Paramoeba* sp.



Pathology associated with *Paramoeba* infections

The outstanding lesion in amoebic gill disease is a severe mucoid branchialitis. Histologically, this branchialitis is characterised by uneven epithelial metaplasia, loss of secondary lamellae, gill fusion, mucus formation, mononuclear cellular reaction and in some cases haemorrhage (Munday *et al.*, 1993) (Plate 5). The associated hyperplasia leads to clubbing and fusion of the secondary lamellae and the formation of large vesicles or cysts (Plate 3;Plate 4) (Munday *et al.*, 1990). Plate 6 shows normal gill structure.

Plate 3 Gill pathology caused by amoebic gill disease-hyperplasia leads to clubbing and fusion of the secondary lamellae and formation of large vesicles x75

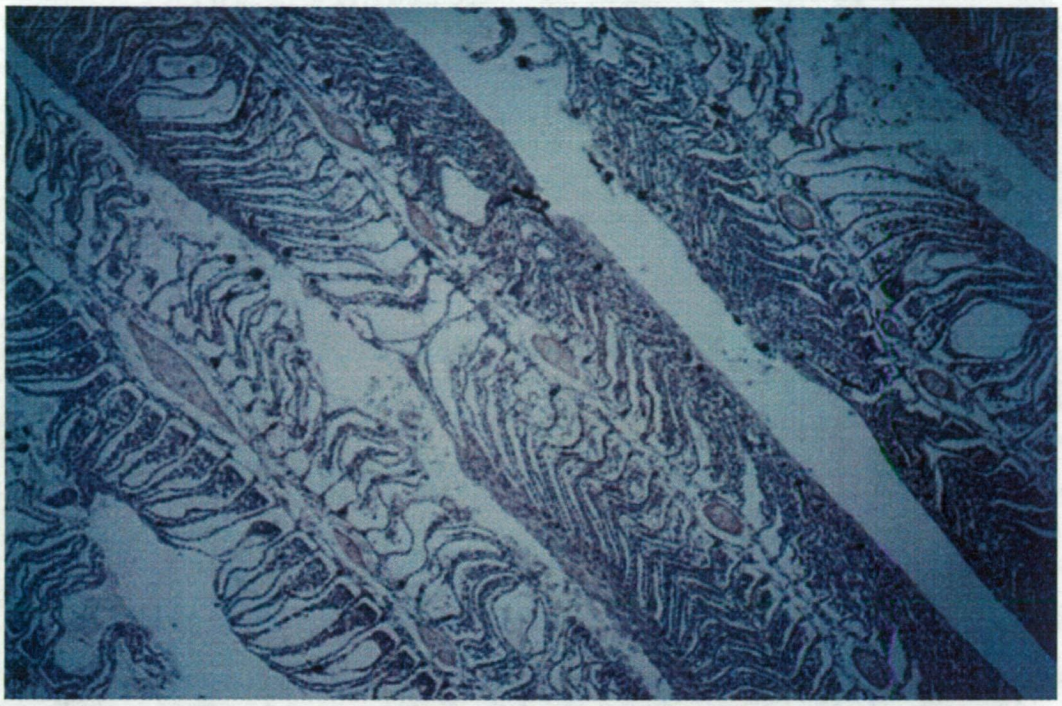


Plate 4 Gill pathology caused by amoebic gill disease-note amoebae concentrations along gill and also their inclusion in the large vesicles x100

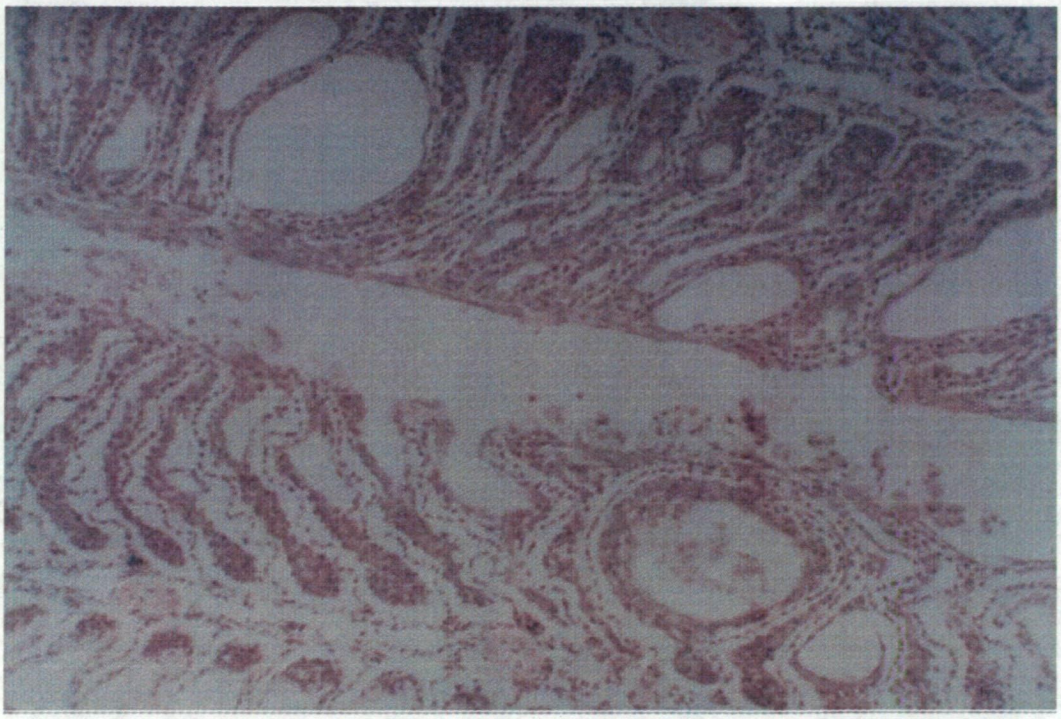


Plate 5 Gill pathology caused by amoebic gill disease-amoebae in situ, note cellular infiltration of gill x400

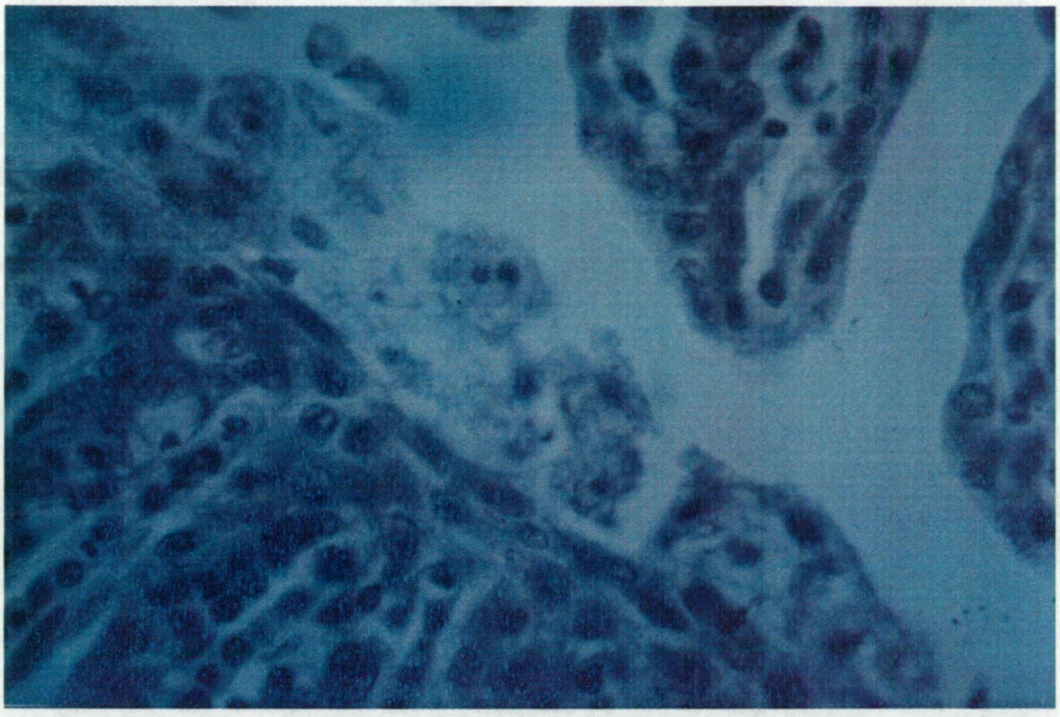
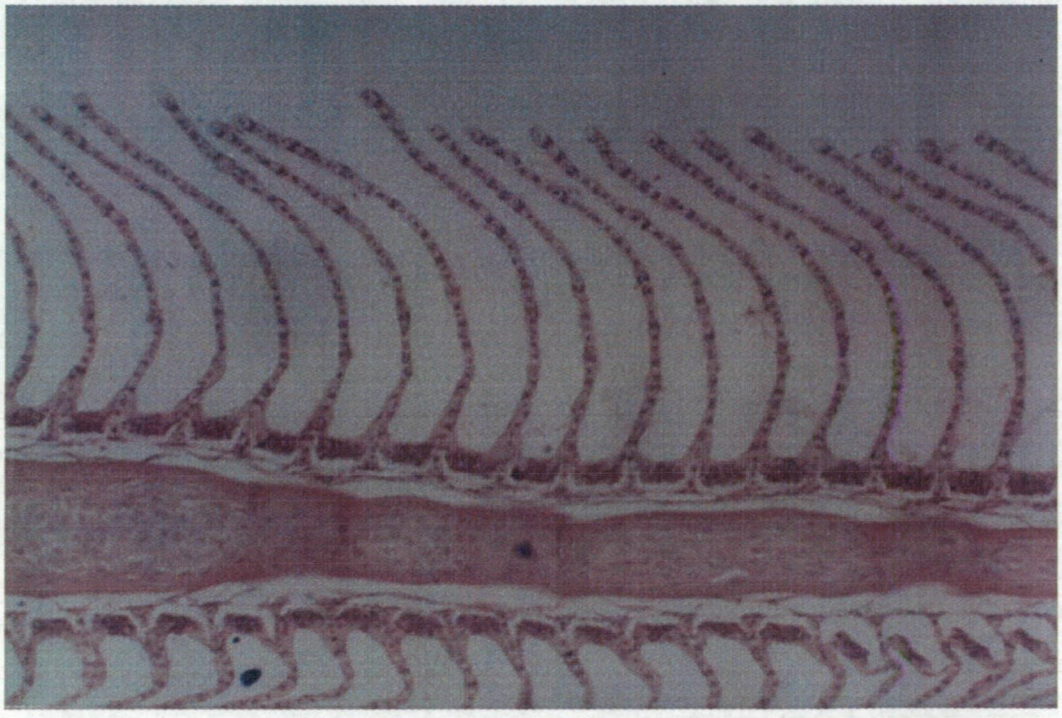


Plate 6 Normal gill structure x200



The lesions of AGD may be found anywhere along the length of the primary lamellae (Munday *et al.*, 1990) with the highest concentrations of lesions occurring on the upper region of the gill arch (Adams, 2000). Microscopically, it is possible to see eroded areas on the lamellae where the organisms attach and ultramicroscopically, pseudopodia of the amoebae can be seen penetrating cell junctions and cavities at the surface of degenerating epithelial cells (Munday *et al.*, 1990). In these areas, actively secreting mucous cells are numerous, both at the surface and deep within the hyperplastic epithelium (Munday *et al.*, 1990; Zilberg & Munday, 2000a). In contrast, chloride cells are reduced in number in the affected areas (Munday *et al.*, 1990). In some cases, sections of hyperplastic tissue can be seen sloughing off with the *Paramoeba* attached to it. In these cases gill lamellae are visibly damaged with exposed blood vessels or entirely lost (Zilberg & Munday, 2000a).

A notable feature of the associated pathology is the infiltration of neutrophils in the hyperplastic epithleium and filamentary connective tissues. As the disease progresses these become mainly of a mononuclear type and in fishes that are recovering, particularly rainbow trout, mononuclear nodules can be seen along the primary lamellae and basal interlamellar tissues (Munday *et al.*, 1990).

Epidemiology and treatment of AGD of salmonids in Tasmania

AGD is not a consistent threat at all times of the year. Epidemiological studies suggest that this most probably relates to environmental factors such as salinity and temperature (Munday *et al.*, 1993, Clark & Nowak, 1999) and the management practices of moving fish to sea and freshwater bathing (Munday *et al.*, 1990).

Although amoebae can be found on the gills of salmonids during winter and spring, clinical disease is usually only seen when water temperatures exceed 12°C (Munday *et al.*, 1990). A recent study by Clark & Nowak (1999) reported the occurrence of AGD in water temperatures of 10.6°C, but this was preceded by higher temperatures.

Even more marked is the affect of salinity with clinical disease only becoming established when salinities approach 35‰. The failure of a major field trial due to a large freshwater spike keeping salinities low was mentioned in the introduction. However, in the study by Clark & Nowak (1999), disease was reported at 7.2‰ for a very limited period of time and residual infection due to preceeding periods of the high temperatures and salinities explain this anomaly.

AGD is currently treated and controlled by the use of freshwater baths and/or brackish water culture sites (Zilberg, Findlay & Munday, 2000). To bath fish, whole cages are usually brought to shore and fish are transferred to land-based tanks or to

floating envelopes filled with freshwater at a maximum salinity of 4‰, but preferably less (Plate 7) (Munday *et al.*, 1990). The optimal time period to hold these fish in freshwater is between two and three hours (Findlay & Munday, 1998) although in practice these periods may exceed eight hours (Plate 8) (Bender[6], *pers. comm.*, 2000). After bathing, the fish are moved into a seapen (Plate 9) and towed back to location on the lease (Plate 10). More recently, one company has begun using platform cages where bathing can be done on-site.

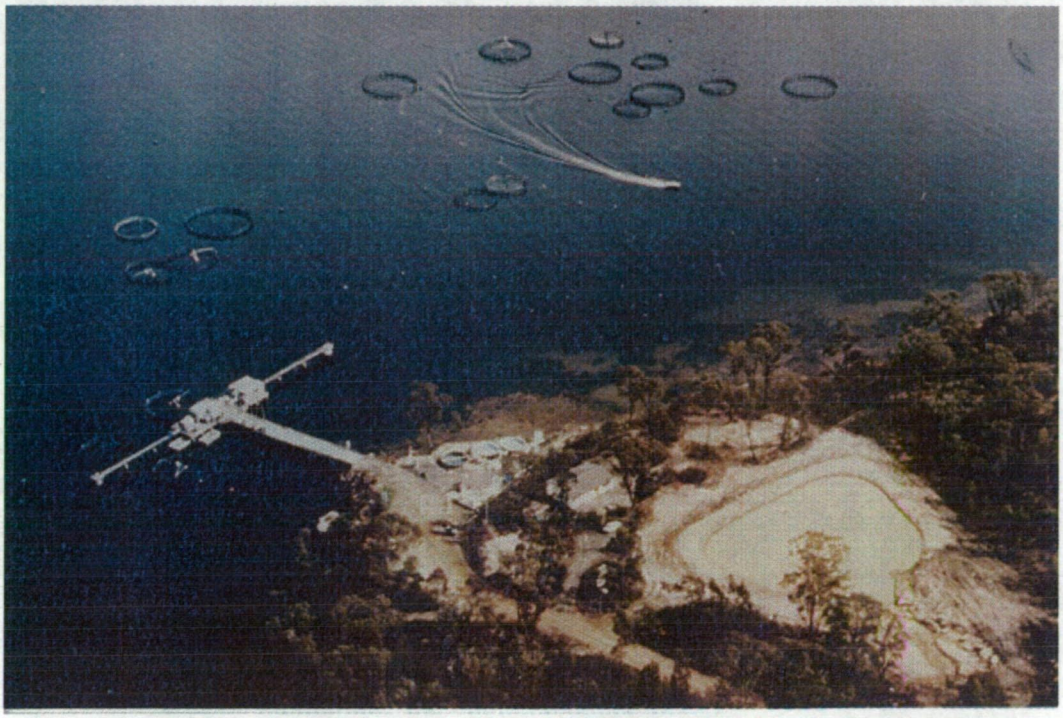
[6] Bender, P. Huon Aquaculture, Tasmania

Plate 7 Atlantic salmon being moved to the freshwater bath

Plate 8 Atlantic salmon infected with AGD are held in freshwater baths for two to three hours

Plate 9 **After two to three hours the bathed fish are returned to their seawater pen via hydraulic lift**

Plate 10 **Aerial photo of Tasmanian salmon farm. Cages are set in designated patterns for husbandry and management reasons. The large dam provides freshwater for AGD bathing.**



Munday *et al.*, (1990) reported that freshwater treatment acts in three ways, namely:

- to reduce the numbers of amoebae on the gills;
- to remove the seawater stable mucus that covers the gills; and
- to reduce any hypernatraemia which may have developed.

CHAPTER 2

GENERAL METHODS AND MATERIALS AND FUNDAMENTAL PROTOCOLS

Contents

The following chapter describes the methods and materials that have been used on more than one occasion and also those that were fundamental to many aspects of this research.

AGD CHALLENGE TRIALS

Atlantic salmon weighing between 100 -200 g were used throughout this study. All fish were naive in relation to AGD, having been maintained in freshwater in 4000l Rathburn tanks connected to individual biofilters (Plate 11). The water temperature was maintained at 14°C via an automated temperature probe connected to a heat exchanger and pump. When these naive fish were needed for challenge experiments they were acclimatized to seawater over a ten day period. All fish were identified by colour coded Hallmark® tags which were inserted before the acclimatization period.

Plate 11 Experimental tanks- 4000l Rathburn holding tanks with biofilter and sump located to the side. A temperature probe sits inside the tank at all times, with heat exchanger situated in the rafters above

Salmon were infected with *Paramoeba* sp. by cohabiting them with fish that had AGD, following the advice of Searle[7] (*pers. comm.*, 1988).

With the exception of losing three tanks of fish due to failed heat regulators and eutrophication of the water through unapproved overfeeding, water parameters were maintained within the optimal range for Atlantic salmon. In a number of instances estuarine water, with salinities ranging from 20-28‰, was delivered by the contractor instead of full salinity seawater (ie 35‰). On one occasion this resulted in the loss of AGD.

For freshwater tanks salinity was always below 2‰, temperature was held at 14°C with a range between 13.6-14.8°C, dissolved oxygen (DO), pH, ammonium and nitrate levels were maintained within acceptable levels.

For seawater tanks the salinity was maintained above 32‰, depending on the need at the time (see protocol for establishment of AGD infection) and temperatures were

[7] Searle, L. Marine Research Laboratories, Taroona, Tasmania

maintained at between 11.2-16.7°C. DO, pH, ammonia and nitrates were maintained within acceptable levels.

All experiments conducted as part of this project were done with the approval of the University of Tasmania Ethics Committee for Animal Experimentation.

Quantification of AGD lesions

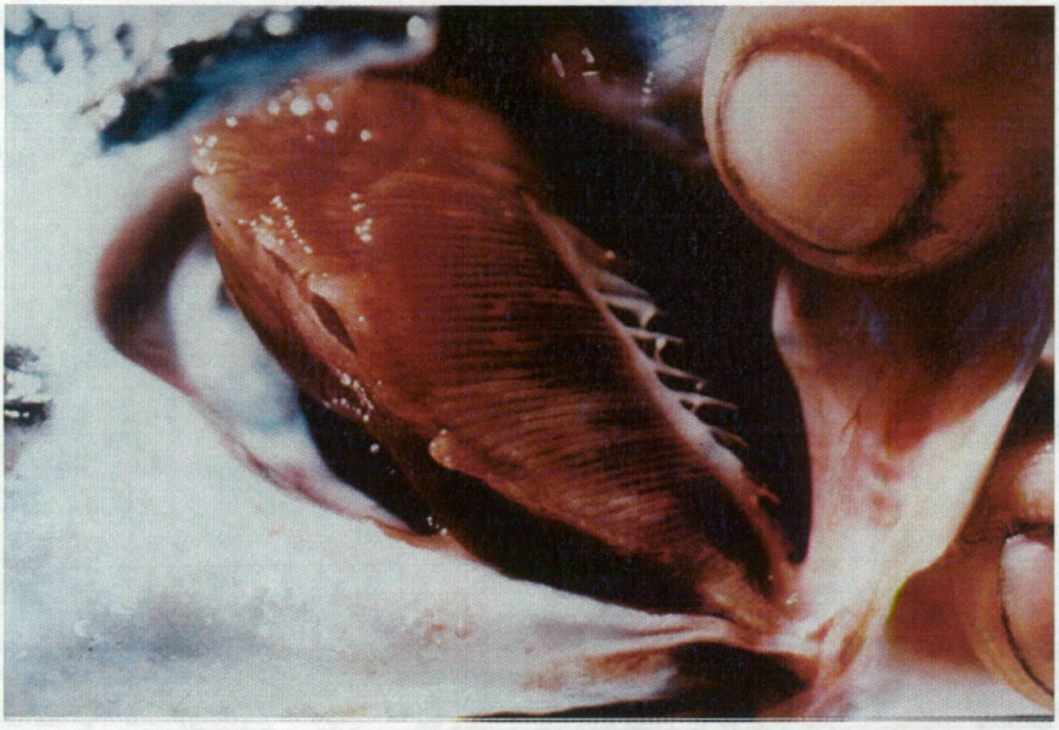
For each experimental AGD challenge, the number of AGD lesions on the first gill arch (including left and right sides) of each fish included in the trial was quantified weekly using a system modified from Alexander (1991).

Once weekly for four weeks post exposure, all fish included in the trial were removed from the trial tank and held in a separate tank. Fish were then lightly anaethetised and the number of lesions present on the left and right first gill arch were counted (Plate 12).

A gill lesion was defined as a focal mucoid patch, white to cream in colour. The size of the lesions ranged from just visible to the naked eye to diffuse large patches covering many gill filaments and containing more than one focal point. In the initial stages of infection mucoid patches were easily distinguishable as separate lesions but as the disease progressed focal patches began to coalesce and form large areas of excess mucus. In these cases clearly visible focal points within diffuse areas of hyperplasia were present. Each focal point was counted as a one patch.

It was recognised that this method of lesion quantification was at risk of being subjective so each fish was identified with an individually numbered tag, as mentioned previously, to allow blind counts to be conducted using two different methods. The first was for three people to count lesions on the same group of fish and the second was for the same person to count lesions on selected fish twice but not consecutively. There was a consistency rate of 96.5% between all counts, with the maximum percentage difference of 3% in the number of lesions detected. Thus, it was established that this method of lesion quantification is repeatable and therefore an appropriate method for studying experimental AGD. A subsequent study conducted by Zilberg & Munday (2000) has also validated this method.

Plate 12 Mucoid lesions on the gills of Atlantic salmon infected with AGD- the lesions on this fish have began to coalesce



The presence of *Paramoeba* associated with the lesions was confirmed by microscopic examination of wet preparations of gill scrapings using both bright and dark field illumination. Paramoeba infection was further confirmed at various intervals throughout the study by immunofluorescent antibody test (IFAT).

IMMUNOFLUORESCENT ANTIBODY TEST

Immunostaining is a sensitive immunological technique that can be used to detect antigen in fish. The indirect fluorescent antibody test (IFAT) was used to periodically confirm the presence of *Paramoeba* sp. in gill tissue samples and smears. Tests were conducted to establish the correlation between the presence of gill patches and *Paramoeba* sp.

The IFAT requires the use of a primary antibody followed by an anti-antibody IgG. The methods and materials used for the production of these are found below.

Initially, gill smears were prepared and heat fixed. The primary antibody, rabbit anti-*Paramoeba*, diluted to the appropriate concentration in IF antibody diluent was applied. The slides were incubated at 37°C for 60 minutes in a humidified incubator before being rinsed with PBS for five minutes. The slides were dried and a fluorescein isothiocynate labeled sheep anti-rabbit IgG, diluted to the appropriate concentration in PBS was overlaid. The slides were incubated as before for 45 minutes and rinsed twice more as previously described. After being mounted in alkaline buffered glycerol they were observed, at 100x magnification using an ultra-violet microscope.

IFAT- preparation of antibody

Antisera were raised by intravenous injection of rabbits with sonicated *Paramoeba*. Injections ranging from 300 000 to 3 million amoeba were administered at three day intervals for three weeks. No adjuvant was used. Antisera were collected four weeks after the last injection.

To determine how specific the antibody was, pure cultures of four different non-Paramoeba species were reacted with the antisera using an anti-rabbit fluorescent label. Antisera were tested at a low dilution of 1:100. A negative control of phosphate buffered saline was also included. Slides were examined by epifluorescent UV microscopy for the presence of labelled amoeba and scored as either a positive or a negative.

To determine if the antiserum to the strain of *Paramoeba* that was injected into the rabbit was specific for AGD-associated *Paramoeba* isolates in general, eight isolates were reacted with the original strain of *Paramoeba* using an anti-rabbit fluorescent label, as above.

CULTURE OF *PARAMOEBA* SP.

Maintenance of cultures

Many species of amoeba grow readily on solid phase substrate with malt-seawater-yeast (MSY) agar being the most common (Kalinina & Page, 1992). *Paramoeba* sp. was cultured as described by Howard, Carson & Lewis (1993).

The growth medium was made by mixing 75% 0.2um filtered seawater and 25% distilled water with 0.1% w/v yeast extract, 0.1% w/v malt extract and 30% w/v agar (Oxoid No.1). The solution was autoclaved at 121°C for 20 minutes and allowed to

cool to 60°C before the antifungal pimaricin (Sigma) was added at a final concentration of 21.5µg per ml of agar.

The molten agar was then poured into 10cm diameter bioassay dishes (Nunc, Denmark) and allowed to solidify overnight at room temperature. The plates were seeded with 2ml of *Xanthomonas maltophilia* suspension (concentration 1.5 x 10^8 cells.ml^{-1}) and allowed to dry for one hour. *Paramoeba* sp. cultures were initiated by placing agar squares that had been freshly excised from pure cultures obtained from Mt. Pleasant Animal Health Laboratories, Prospect, Tasmania on the pre-seeded plates. Cultures were maintained at 20°C in sealed containers to minimize evaporation. The plates were subcultured every four weeks.

As required, large numbers of *Paramoeba* sp. were grown using 23x23cm square culture plates (Nunc, Denmark). The only difference to the culture technique described above was that these plates were overlayed with sterilised seawater and the temperature adjusted according to the requirements for the experiment to be conducted.

Xanthomonas maltophilia culture

Original cultures of *X. maltophilia* were obtained from Mt. Pleasant Animal Health Laboratories. Liquid cultures were grown by seeding nutrient broth (Oxoid No. 2) with 1-two bacterial colonies removed from cultures maintained on blood agar plates. The liquid cultures were incubated at 37°C for 72 hours before the bacteria was harvested by centrifugation. The collected cells were washed three times in PBS before being aliquoted and stored at 4°C.

PHAGOCYTOSIS ASSAY

The ability of fish phagocytes to ingest particles is usually demonstrated with an *in vitro* phagocytosis assay. These tests are expensive and labour intensive. To overcome this an *in vivo* phagocytosis assay was devised that is very cheap to run and reduces the factors that result in high individual variation.

Preparation of yeast cells

Suspensions of cells of the yeast *Saccaromyces cerevisiae* (10^8 cells.ml^{-1}) were prepared in phosphate buffered saline (PBS) (pH 7.4). The equivalent amount of 0.8% congo red was added to the yeast suspension before it was autoclaved at 121°C for 20 minutes. This yeast cell solution could be stored refrigerated for up to 72

hours. Before use, the yeast cells were washed five times in sterile PBS (3 minutes at 300g) and resuspended in the initial volume of PBS.

In vivo phagocytosis methodology

Fish were anaesthetised with 40mg.L^{-1} benzocaine (10% wv-1 ethyl-4-aminbenzoate in acetone) to Stage III or surgical procedure anaesthesia as described by Ross and Ross (1984). Each fish was weighed and injected with 0.1ml per 100g body weight of the yeast solution via the caudal vein. The fish were revived and held in 400l plastic tanks supplied with oxygen for two hours. After the holding period the fish were anaesthetised once again and bled by severing the caudal vessels.

Preparation of the cell suspension

The head kidney was removed and placed on a stainless steel mesh sieve (0.3mm) in a petri dish containing 5ml of PBS. The kidney tissue was pushed through the mesh and the resulting homogenate passed through a loosely packed glass wool syringe column to remove tissue and cellular debris and red blood cells. The suspension was allowed to settle for 15 minutes and then gently layered upon 2ml of Histopaque 1077 (Sigma). The layered cell suspension was centrifuged for 15 minutes at 400*g*.

Following centrifugation, the white blood cell interface layer was removed and washed in 3ml PBS (three minutes at 200*g*). The supernatant was decanted and the pellet resuspended in 0.5ml of PBS. The solution was vortex mixed and a drop placed on a glass slide and covered with a coverslip. Cell viability (>95%) was confirmed using trypan blue exclusion (0.5% trypan blue solution). Viability was tested as confirmation for what I believed already to be the case that phagocytes were viable and able to phagocytise and that there was not a large number of *in vivo* senescent cells as a result of the treatments.

Phagocytic cells, defined as neutrophils and macrophages and identified accordingly, were counted until 100 cells that had consumed yeast had been recorded. The number of yeast cells in each phagocyte was counted and the phagocytic index and phagocytic capacity and phagocytic activity calculated as follows:

- Phagocytic index (PI) is the total number of yeast cells consumed, divided by the number of consuming phagocytes.
- Phagocytic capacity (PC) is the total number of phagocytes containing a given number of yeast cells divided by the total number of phagocytes containing any yeast.

- Phagocytic activity (PA) is the number of consuming phagocytes divided by the total number of phagocytes counted.

STATISTICAL ANALYSIS

One-way and two-way analysis of variance tests, Tukey's (HSD) and LSD tests were performed using the general linear models procedure of the SAS system for Windows 6.11. Data were considered to be significant at the 5% level ($p<0.05$). Regression analyses were performed using the statistical package of Microsoft Excel 97.

CHAPTER 3

EXPERIMENTAL REPRODUCTION OF AMOEBIC GILL DISEASE IN ATLANTIC SALMON

Contents

INTRODUCTION

There is little doubt that the *Paramoeba* sp. that can be isolated from the gills of Atlantic salmon and rainbow trout in Tasmania is the aetiological agent of AGD. However, Koch's postulate has not been fulfilled in relation to infecting susceptible animals with the cultured organism.

Irrespective of whether Koch's postulate had or could be fulfilled, it was necessary to have a method of consistently reproducing the disease in an experimental situation in order to effectively study the response of Atlantic salmon to AGD. Two approaches were used.

One method was to attempt to reproduce AGD using cultured *Paramoeba* sp.. This approach had been tried by Kent *et al.* (1988), Howard *et al.* (1993) and Searle (*pers. comm.*, 1988) without success. While it is most likely cultural conditions (eg type and variety of 'food' items) that cause *Paramoeba* to lose their virulence when cultured, there were practical constraints that could have accounted for the above failures to reproduce the disease and my study addressed some of these. In the trial described here, varying concentrations of *Paramoeba* were used in the water column and the organism was subjected to different temperature regimes in an effort to

mimic the natural situation. Also, I instituted a more stringent experimental design protocol than the above-mentioned researchers.

The other method was to expose naïve fish to infected fish (cohabitation method). This was based on the serendipitous observation of Mr Lance Searle of the Tasmanian Department of Sea Fisheries that when uninfected trout came in contact with infected fish they developed AGD. This observation was subsequently experimentally verified by Howard *et al.* (1993) using rainbow trout. However, they only maintained experimental disease for 14 days. For my work it was necessary to reproduce the disease in Atlantic salmon and maintain it for an extended period of time.

METHODS AND MATERIALS

The following methodology development studies were undertaken prior to and during the main part of my research program.

Cultured *Paramoeba*

The experiment reported here was designed to test the infectivity of cultured *Paramoeba* sp. to Atlantic salmon.

Large numbers of *Paramoeba* sp. were cultured using the method described in Chapter 2. *Paramoeba* were harvested by washing the surface of the plates with sterilised seawater. Two 30 litre tanks of amoebae suspension at concentrations of 5000 amoebae/mL and 15 000 amoebae/mL were set up, with one treatment at each concentration using *Paramoeba* maintained at 20°C and the other with *Paramoeba* acclimated to 14°C. All baths were maintained at 14°C and aerated to the extent necessary to keep the amoebae suspended in the water column.

The viability of the organisms to be used in this experiment was established by adding 0.5% trypan blue solution to an aliquot of the *Paramoeba* suspension. The number of viable cells were counted using a haemocytometer and the percentage calculated.

Fifteen 100g Atlantic salmon smolts were added to each bath for two hours. This process was repeated with a second group of fish to increase to 30 the number of fish that had been exposed to cultured *Paramoeba*. It is acknowledged that the ideal experimental design would be to bath all 30 fish for each treatment at once, however resource limitations meant this was not possible.

After the two hour bath, the fish were placed in four separate 4000L fibreglass tanks and maintained using the protocol for the maintenance of *Paramoeba* infection, as described in Chapter 2. Each week, for the four weeks post-treatment, all fish were anaesthetised and all gill arches were examined for signs of amoebic gill disease. In addition to this, each week five fish from each treatment were killed and the left and right first gill arches excised; one arch was fixed in Davidson's fixative for histological examination and the second was examined as a wet preparation using dark and bright illumination microscopy. After microscopical examination a smear was made from each sample and a *Paramoeba* IFAT conducted using the protocol described in Chapter 2.

Wild *Paramoeba*

For the establishment of a model for infection using fish-associated *Paramoeba* it was necessary to maintain a 'pool' of donor salmon with disease and to use these donors to infect experiment fish through cohabitation. The only protocols for experimental AGD infection that had been used prior to this study could not maintain infected fish for longer than approximately 14 days (Howard *et al.*, 1993) and fish that were chronically infected with AGD had never been maintained. Refinement of the method that had been previously used was needed.

Infected fish obtained from Aquatas Salmon Farm, Margate, Tasmania were placed into two 4000l fibreglass tanks. Each tank was connected to an individual biofilter to permit recirculation of the water. The water temperature was maintained by means of a heat exchanger connected to a heat pump with adjustments automatically initiated by an electronically controlled temperature probe.

Initially, 20 naïve fish were introduced to each tank with the donor fish. The temperature was maintained at 14°C and the salinity at 35‰. The level of the disease was monitored by anaesthetising five fish and recording the number and severity of lesions present on the first gill arch. Smears were also made from the gills of each anaesthetised fish so an IFAT could be used to confirm the presence of *Paramoeba* in the lesions. Once sufficient disease was established the temperature was lowered to 12°C. At 12°C the progression of the disease was easily managed with mortalities occurring sporadically. At all times a minimum of 20 fish were maintained with AGD. After five mortalities had occurred from this disease reservoir tank five naïve fish would be added. However, it was found that at 12°C, horizontal transmission was not always facilitated so the temperature was raised to 14°C when additions were made. At 14°C the naïve fish would become infected after four to five days at

which point the temperature would once again be lowered to 12°C. Using this protocol and maintaining a vigilant monitoring system, AGD was maintained in the experimental situation for 18 months.

RESULTS

Cultured *Paramoeba*

The percentage viability of *Paramoeba* used in this experiment was greater than 98%.

No fish that had been exposed to cultured *Paramoeba* using the methods described above developed AGD and no amoeba could be seen on the gills either histologically or microscopically. Further, none of the IFAT tests detected the presence of *Paramoeba* on the gills of exposed fish.

Wild *Paramoeba*

Naïve fish that had been exposed to AGD via cohabitation with donor fish consistently developed AGD. The time to when the first lesions could be seen in naïve fish varied between four to ten days. As the virulence of the *Paramoeba* increased, as discussed below, the time to when the first lesion could be seen decreased.

The *Paramoeba* IFAT provided a reasonably reliable means of confirming that *Paramoeba* were associated with the mucosal gill lesions. In most instances where lesions were seen, the IFAT confirmed the presence of *Paramoeba* sp. The sensitivity of the IFAT appeared to increase as the disease progressed. In fish that were heavily infected the IFAT detected the presence of *Paramoeba* in 100% of the samples taken. However, in the early stages of disease the IFAT did not reliably confirm the presence of *Paramoeba*, even when organisms could be seen under the microscope[8].

More than providing a means for the consistent reproduction of disease, this protocol allowed AGD to be maintained in the experimental situation for long periods of time (up to 18 months in one instance) and the manipulation, albeit in an imprecise manner, of the severity of AGD.

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[8] Since these observations, Zilberg & Munday (2000) have confirmed the relatively low sensitivity of the IFAT, but the reason(s) is not apparent at this stage.

The initial strain of AGD that was obtained from Margate, Tasmania, became very virulent after 18 months continual passage into naïve fish and was causing pathology and death in short periods of time, so the decision was made to acquire another field strain. This strain was maintained using the same techniques described above.

It was noted during the establishment of this infection protocol that fish that had been chronically infected with AGD over the period in which a number of sequential additions of naïve fish had been made, often became resistant to acute AGD infections. It was also noted that if these fish were the only donor fish in the tank when naïve fish were added, it took a longer period of time (ie two weeks instead of one week) for AGD infection to become established in the naïve fish.

Another incidental observation that was made during the establishment of an infection protocol was that the addition of a protein skimmer[9] to a tank holding AGD infected fish resulted in a spontaneous resolution of disease, together with clearing of *Paramoeba* from the gills. This observation was not further investigated.

DISCUSSION

As discussed in Chapter 1 there are at least three species of *Paramoeba* that have been associated with disease in susceptible animals. Of these, Koch's postulate has been fulfilled for only one; *P. invadens*.

There does, however, appear to be some debate in the scientific community regarding the use of Koch's postulate to prove the aetiological cause of disease. As an example, information was recently forwarded to me from Dr Matti Kiupel of the Animal Disease Diagnostic Laboratory, Indiana, USA, suggesting that Koch's postulate is no longer the standard test to prove an aetiology. The molecular updated version of Koch's postulate consists of proof that the organism is associated with a lesion/disease, to identify this organism (isolating it or confirming sequence data etc) and reproducing the disease somehow....."

[9] Put simply, a protein skimmer consists of a reaction chamber where the water is mixed with small air bubbles. As the bubbles rise through the water, protein molecules are attracted to the water/air interface and become attached to the air bubble, rising to the top of the reaction chamber where they are collected. This removes the proteins from the system before the bacteria can digest them.

The opposing view is that fulfilment of Koch's postulate is the only conclusive proof of aetiology. Dr Douglas Gregg of the Foreign Animal Disease Diagnostic Laboratory, USA commented 'that while opinions based on comparative pathology, electron microscopy, comparative immunology, immunohistochemistry, and *in-situ* hybridization...are interesting and cannot be ignored, the final answer on the aetiology of a disease awaits the fulfilment of Koch's postulate (in more than one laboratory). Until the agent can be isolated *in vitro* and disease then reproduced, most scientists should be skeptical' (Gregg, 1996).

Where disease has been reproduced using organisms isolated from diseased animals, such as has been done by Zilberg, Gross & Munday (2000) who reproduced disease using *Paramoeba* sp. isolated from infected gills, it appears that the fulfilment of Koch's postulate in relation to the *in vitro* isolation of the organism and subsequent reproduction of disease may be outdated. It is suggested that microbiological and molecular constraints in the period Koch's postulate was first accepted as the standard for proof of the aetiology of disease led to the conclusion that *in vitro* culture was the only way to 'isolate' a given pathogen. Given the developments in these fields, it is intriguing that the strict application of Koch's postulate has persisted. Certainly in the instance of AGD, the aetiological agent has been defined by the above authors without strict fulfilment of Koch's postulate.

Even so, this does not eliminate the possibility that some other co-factor such as the presence of a virus or bacterium or another predisposing factor, may be required along with *Paramoeba* sp. Certainly other workers in the field including Kent et al (1988), Munday et al (1990) and Nowak & Munday (1994) have suggested that pre-disposing factors may influence the development of AGD. However, more recent work conducted to Zilberg & Munday (2000b). demonstrated that *Paramoeba* sp. will attach to healthy epithelium suggesting that the gills do not have to be damaged in order to allow the infection to occur and that the attachment of *Paramoeba* sp. triggers epithelial lesions in healthy gills. Also, Zilberg, Gross & Munday (2001) reproduced the disease with isolated *Paramoeba* sp.. Therefore, if a virus or a bacterium is involved it must by a commensal of the amoeba, something yet to be researched.

As previously stated, whether Koch's postulate had, could or needed to be fulfilled it was necessary to optimise an infection regime as well as develop a protocol that allowed AGD to be maintained in the experimental situation.

In my trials the only difference between the two procedures for the establishment of AGD was that cultured organisms were used in one and wild *Paramoeba* released

from the gills of infected fish in the other. The materials and methods described above for the cultured *Paramoeba* trials were the same as those used in the cohabitation study. Naïve fish from the same group of post-smolts were used as the challenge fish in both trials, the same batch of seawater was used and the temperature regime used in the cultured *Paramoeba* trial mimicked that of the cohabitation challenge. The cohabitation protocol consistently led to the reproduction of disease; the protocol using cultured *Paramoeba* did not. Although all practical constraints associated with reproduction of AGD using cultured organisms have not yet been ruled out it, given the mounting evidence, these seem to be an unlikely reason for the failure of cultured organisms to reproduce disease. Further, Zilberg *et al.* (2000) demonstrated that only 230 *Paramoeba* sp. per litre harvested from infected fish and directly added to a bath with naïve fish were needed to establish infection. Thus, even if less than 10% percent of the cultured *Paramoeba* sp. used in this study were virulent, as has been demonstrated in certain circumstances for cultures of *Aeromonas salmonicida* (Ishiguro, Kay, Ainsworth, Chamberlain, Austen, Buckley & Trust, 1981), the minimum infective dose was clearly exceeded.

It appears most likely that cultured *Paramoeba* are missing out on 'something' or getting 'something' in the culture process that renders them non-virulent. The phenomenon of loss off or decreasing virulence in pathogenic organisms is not uncommon. A lot of the knowledge on virulence and virulence factors of pathogenic organisms is the result of studies using bacterial pathogens. However, there are number of studies on protozoan pathogens, particularly *Entamoeba histolytica* that may indicate reasons for the loss of virulence that is seen in cultured *Paramoeba* sp..

In 1960, Vincent & Neal (1960) described the gradual decrease in virulence of *E. histolytica* maintained *in vitro*. These workers also demonstrated that virulence could be restored if the cultured organisms were passed through host cells. Although virulence was never completely lost during the Vincent and Neal study, it indicates that 'something' was not right in the *in vitro* environment. Subsequent studies by, *inter alia*, Wittner & Rosenbaum (1970); Phillips, Diamond, Bartgis & Stuppler (1972) and Bos & Hage (1975) suggested that the virulence of *E. histolytica* could depend on an episome-like factor that required direct contact with live bacteria in order to be expressed. Given that *Paramoeba* sp. has always been cultured using *Xanthomonas maltophila* as a source of nutrition, it may be that this bacteria does not provide the necessary properties in order for virulence to be expressed in the amoeba (in this respect it would be useful to determine the sugar moieties on *X. maltophila*). This scenario has certainly been demonstrated for *E. histolytica*. Padilla-Vaca, Ankri, Bracha, Koole & Mirelman (1999) demonstrated that *E. histolytica* grown with a galactose (Gal) /N-acetyl-*D*-galactosamine (GalNAc) binding strain of *E. coli* showed markedly reduced adherence and cytopathic activity. Specific lectin gene

probes revealed a decrease in the transcription and expression of the Gal/GalNAc light subunit. This effect was not observed when *E. histolytica* was grown with a mannose-binding strain of *E. coli*. These workers concluded that the light subunit of the amoebic lectin is involved in the modulation of the parasite adherence and cytopathic effect.

One common theme for many pathogenic organisms is the use of microbial lectins for colonization and invasion. In this regard, Mirelman[10] (*pers. comm.*, 2000) stated that most amoeba infections begin by a lectin mediated binding of the pathogen to a host mucosa and the mucus of the gills is an appropriate template. He further commented that it is likely that the carbohydrate affinity will be some glycoconjugate rich in galactose or *N*-acetylgalactosamine, as has been found for many of the amoeboids.

In this regard, Ravdin & Guerrant (1981) established that adherence and subsequent contact dependent cytolysis of the chinese hamster ovary (CHO) cells were nearly completely blocked by galactose or *N*-acetyl-*D*-galactosamine. This galactose inhibition of amoebic adherence was also shown to extend to human erythrocytes and neutrophils, certain bacteria, human colonic mucosa and rat and human colonic mucins (Petri, 1991).

Of particular interest to the research on AGD are the studies by Li, Becker & Stanley (1988) and Saffer & Petri (1991) that demonstrated galactose in a β1-4 linkage with N-acetylglucosamine was the preferred ligand for the amoebic lectin and that N-acetylglucosamine is a significantly more potent inhibitor of the amoebic lectin than is galactose. These workers suggested that the galactose specific lectin that occurs on the amoeba may bind N-acetylglucosamine with a much higher affinity than galactose. The details of why this finding in *E. histolytica* may provide evidence of the mechanisms for resistance of Atlantic salmon to AGD will be discussed in a later chapter.

It is important to note that not only are there moieties on the amoeba that may enhance or facilitate adherence but that there may be lectins on the gills that display adherence properties towards sugar moieties on the amoeba. These interactions are very complex and it is likely, if not probable, that there is more to adherence and cytopathology than the galactose-specific lectin alone. It has been shown for *E. histolytica* that isolates can be grouped into pathogenic and non-pathogenic zymodemes on the basis of isoenzyme analysis (Petri, Jackson, Gathiram, Kress,

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[10] Mirelman, D. Weismann Institute, Israel

Saffer, Snodgrass, Chapman, Keren & Mirelman, 1990). Similar isoenzyme analysis of *Paramoeba* sp. is warranted.

Other distinctive features of *E. histolytica* that may relate to, or indicate, pathogenicity include the thickness of the cell surface coat. El-Hashimi & Pittman (1970) and Lushbaugh & Miller (1974) showed that invasive forms of amoebas have a thicker and more prominent surface coat. A thicker surface coat may indicate an increase in the moieties specific to adherence and cytopathology.

The overall surface charge of a given strain of amoeba may also indicate pathogenicity. Trissl, Martinez-Palomo & Chavez (1976) demonstrated that low virulence strains of *E. histolytica* could be labelled with cationic ferritin at neutral pH and that these strains had an overall negative electrical charge at the cell surface. Virulent strains of *E. histolytica* did not have electrophoretic mobility at neutral pH and did not bind cationic ferritin. It is interesting that Martin (1987) showed that *Paramoeba pemaquidensis*, the presumed agent of AGD, required a minimum surface negative charge for adhesion to substrata.

All of the above variables are likely to be influenced by artificial culture and need to be addressed in a logical sequence if Koch's postulate needs to be fulfilled.

Just as the attenuation of virulence in pathogenic organisms that are cultured so often happens, the increase in virulence following passage through susceptible hosts is equally well reported. Eldar, Bejerano, Livoff, Horovitcz & Bercovier (1995) and Mateos & Paniagua (1996) demonstrated an increase in the virulence of *Aeromonas hydrophila* and *Streptococcus* sp. respectively with serial passage through susceptible hosts. This study reports a similar situation for *Paramoeba* sp. For a large proportion of this study, one strain on *Paramoeba* sp. was maintained. However, as discussed previously, after continual passage into naïve fish, this strain was causing significant pathology and mortality in very short periods of time, so it was decided to obtain another field strain to continue the challenge trails. Although the mechanisms for the increase in virulence are unclear, some insight may be gained from evidence relating to the attenuation of virulence. Presumably, an increase in virulence would be expressed as a result of a changed composition of the moieties associated with the surface coat.

Some comment on the possible reasons why a protein skimmer may have resulted in resolution of the disease and removal of *Paramoeba* from the gills is needed.

If in fact the composition of the bacteria to which the *Paramoeba* are exposed regulate virulence factors then a change in the protein composition of the tank

environment, as a result of the protein skimmer, could result in a decrease in transcription and expression of the adherence lectin, as has been demonstrated for *E. histolytica*, or an inhibition of adherence to the gill as a result of the presence of a preferred ligand. Alternatively, as *Paramoeba* are shed from the gills in mucus particles (Zilberg & Munday, 2000a) the process may simply have removed the organisms from the water column to the extent that a threshold population was no longer present in donor fish and an infective dose was not available to naïve fish.

The application of the hypotheses presented above, in relation to the Atlantic salmon acquiring resistance to AGD will be discussed in later chapters.

While a protocol using cultured organisms for the reproduction of AGD would provide a means of carrying out highly controlled tanks trials, irrespective of season, as well as reducing the monetary and time costs because 'donor' fish would not be needed, the development of a protocol which allows the maintenance of 'donor' and experimental fish indefinitely has been a major achievement. Without this protocol effective and robust *in vivo* experimental studies of AGD could not have been undertaken.

CHAPTER 4

DEMONSTRATION OF RESISTANCE TO AMOEBIC GILL DISEASE IN ATLANTIC SALMON

Contents

INTRODUCTION

During the early years of amoebic gill disease occurring in farmed salmonid stocks in Tasmania it was noted that fish often experienced three waves of infection coupled with the need for three rounds of freshwater bathing. There was much anecdotal evidence suggesting that fish that had been previously infected with AGD and treated with a freshwater bath appeared to be relatively resistant to re-infection. Often, the third bath was carried out in response to only a small percentage of the fish relapsing. Thus, it appeared that the success of the freshwater baths was dependent on fish acquiring immunity.

Atlantic salmon have been shown to respond to *Paramoeba* antigens, both when immunized parenterally with killed and live organisms, and when subjected to severe natural and experimental infections (Akhlaghi, 1994). As well as humoral antibodies, it is possible that local (gill mucus) antibodies may be formed, and as with bacterial gill disease (Lumsden, Ostland, MacPhee, Derksen & Ferguson, 1994), these may be protective. The trial described here was designed to test this possibility, as well as provide firm evidence for the development of resistance in fish that had previously suffered from AGD.

As well as testing whether AGD induced a specific immune response, a phagocytosis assay was conducted on infected fish during this challenge trial in order to detect if the nonspecific defence response was influenced by the infection.

As it had been reported that osmoregulation can be compromised in fish severely affected with AGD (Munday *et al.*, 1990), the opportunity was taken to record the plasma osmolality in fish from each of the experimental groups.

MATERIALS AND METHODS

AGD challenge trial

Initially, two replicates of 50 Atlantic salmon smolts of 100-150g bodyweight (mean 134.6g ± 9.8g) naive for AGD, having been maintained in freshwater, were placed in tanks maintained at 14°C with 12 Atlantic salmon post-smolts infected with AGD. After four weeks all fish displayed lesions and mortalities had occurred. With the exception of eight fish from each tank (which were used in a phagocytosis assay described below), the surviving fish were transferred to a freshwater tank where they recovered quickly. After a further four weeks, the fish were divided into two groups and placed into seawater tanks with equal numbers of naive fish. Infection was initiated by adding twenty post-smolts with AGD to each tank. The severity of the resultant AGD was quantified as described previously (Chapter 2) and the number of mortalities in each group recorded.

Osmolality

During the post-exposure weekly gill counts, blood samples were taken, using a heparinized tuberculin syringe fitted with a 21 gauge needle, from the caudal vessel of ten fish from each of the infected, naïve, control and donor groups. The blood was immediately centrifuged, the plasma separated and stored on ice until use as soon as

practicable on the same day. The plasma osmolality of each sample was measured by vapour pressure osmometry (Advanced 505 osmometer).

Detection of anti-*Paramoeba* antibodies

At the completion of the above challenge, serum and mucus was collected from each of the remaining fish in each of the four groups and assayed for the presence of anti-*Paramoeba* antibodies.

Serum collection

Fish were anaesthetised as previously described (Chapter 2). Each fish was turned into the supine position and held in a cradle with head elevated. Prior to the fish being bled from the caudal vessel, the body mucus was removed with paper towelling and the fish wrapped in paper towels to prevent contamination of the gill mucus. As much blood as possible was collected from the caudal vessel using a 21-gauge needle and a 15ml syringe; this was allowed to clot overnight at 4°C. The samples were centrifuged at 1500*g* for 15 minutes, the serum aliquoted and stored at -80°C until use.

Mucus collection

With the fish's head still elevated the tail was severed at the peduncle region and then the heart was exposed. The gills were perfused with heparinized saline by direct puncture of the bulbous arteriosus or ventricle using a 21-gauge needle and a 50ml syringe. Depending on the size of the fish complete perfusion generally occurred after about 30 seconds and 20-30ml of saline had been injected. Perfusion was judged as being complete when the gills were blanched white and the fluid flowing from the caudal vessels was clear. The gills were excised, weighed and immediately placed in an antibody extraction cocktail as described by Lumsden, Ostland, Byrne & Ferguson (1993). In short, this consisted of 0.85% saline, 2mM phenylmethylsulphonyl-flouride (PMSF), 2mM N-ethylmalemide (NEM), 10mM disodium ethylenediamine tetraacetate (EDTA), and 0.02% sodium azide (NaN_3), at 4°C for two hours with occasional shaking. After soaking, the gill tissue was removed and the mucus preparations were centrifuged at 30 000*g* for 30 minutes at 4°C. The resulting supernatants were dialyzed against distilled-deionized water containing 2 mM PMSF and 0.02% sodium azide. Additional debris was removed from the dialysate by centrifugation (1000*g* for 15 minutes). The mucus preparations were lyophilized and stored in airtight containers until use. Each individual sample was resuspended in a volume of PBS with NaN_3 proportional to the wet weight of the initial sample, before the immunoassay was performed.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to determine the specific anti-*Paramoeba* antibody values in serum and mucus samples. The assay used was modified from the ELISAs as described by Akhlaghi, Munday & Rough (1993), Bryant, Lester & Whittington (1995) and Howard & Carson (1994). The incubation intervals, temperature, pH and diluents involved in each step of the ELISA were optimized using previously collected serum samples from fish that had been inoculated with *Paramoeba* antigen and fish that were naïve in relation to *Paramoeba* sp.. This minimized background noise and increased the difference between the negative and positive control sera.

Preparation of amoebic antigen

Paramoeba sp. were cultured as previously described (Chapter 2). The amoebae were harvested from plate cultures by washing with filtered sterile sea water (FSSW) and the contaminating bacteria used as nutrient substrate were mostly removed by centrifugation (1000*g* for 20 minutes in FSSW, x 3). The washed *Paramoeba* cells were then resuspended in 2mls of FSSW and sonicated. The protein concentration of the sonicate was determined and readjusted to give a final concentration of $1 mg.ml^{-1}$. The preparation was then stored at –20°C until use.

Preparation of *X. maltophilia* antigens

The soluble antigen was prepared by washing the bacterial cells that had been harvested from nutrient broth (as previously described in Chapter 2) with sterile distilled water followed by sonication. The protein concentration of the sonicate was determined and the antigen stored at –20°C.

Absorption of *X. maltophilia* antibodies

As the *Paramoeba* harvested for use as the soluble antigen had been cultured using *X. maltophilia* for nutrition, the *Paramoeba* antigen would contain bacterial contaminants. To ensure that the results of the ELISA were due only to the presence of anti-*Paramoeba* antibodies, and not anti-*X. maltophila* antibodies, serum and mucus samples were absorbed with *X. maltophilia* sonicate.

Serum and mucus samples were diluted in PBS (1:50) and mixed with *X. maltophilia* sonicate ($200ug.ml^{-1}$) in a ratio of 1:1 for 11/2 hours at 4°C. The absorbed samples were then centrifuged for two minutes at 10 000*g* and the supernatant collected for

ELISA evaluation. The efficacy of absorption was determined by ELISA, using several different dilutions of antigen and antibody.

General ELISA methodology

Paramoeba sp. and *X. maltophilia* antigens were diluted in coating buffer (50mM sodium hydrogen carbonate, pH 9.5) to a concentration of 5ug.ml^{-1}. The 96 well microtitre plates (flat bottom polystyrene microtitration ELISA plates by Linbro/Titertek) were coated with 100ul of antigen per well and incubated overnight at 4°C. After incubation the antigen solution was flicked off and the wells blocked with PBS/3% casein (100µl/well) for 30 minutes at 25°C. The plates were then washed three times with PBS/0.05% tween-20.

Serum and mucus samples which had been diluted 1:100 and 1:10-1:100, with PBS/1% casein, respectively, were added to the wells (100µl/well) and the plates were incubated at 25°C for 90 minutes. All samples were tested in triplicate. After washing, mouse anti-rainbow trout monoclonal antibody, supplied by Dr Richard Whittington, EMAI, diluted 1:10 in PBS/1% casein was added (100µl/well) and the plates were incubated for 90 minutes at 25°C. Plates were washed three times and rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dako®), diluted 1:1000 in PBS/1% casein was added (100µl/well) before the plates were incubated for a further 90 minutes at 25°C. Plates were washed five times before the colour developing solution, OPD (0.07% o-phenyl-enediamine dihydrochloride in sodium citrate phosphate buffer with 0.012% hydrogen peroxide, pH 5, 100µl/well) was added. The plates were incubated in the dark for five minutes before the reaction was stopped with 3N hydrochloric acid (50µl/well). The optical density was measured at 492 ηm using an automated microplate reader (model EL309). A negative and positive control sample was included in triplicate on each plate.

Correction of optical density readings

All optical density readings were corrected by the plate correction factor (PCF) that follows. Briefly

PCF= (Target O.D. A/Actual O.D. A + Target O.D. B/Actual O.D. B + Target O.D. C/Actual O.D. C + Target O.D. D/Actual O.D. D) /4

Where A, B, C, and D indicate the optical densities for the highly positive, the medium positive, the low positive and the negative respectively. The target optical density refers to the mean optical density for each control over the entire period of testing and the actual optical density is the mean optical density of the triplicated wells.

The positive/negative cut-off was defined as the mean optical density for the negative controls plus three standard deviations (Johnson, Roberts & Munday, 1988). Optical density readings equal to and above this were considered sero/muco-positive.

PHAGOCYTOSIS ASSAY

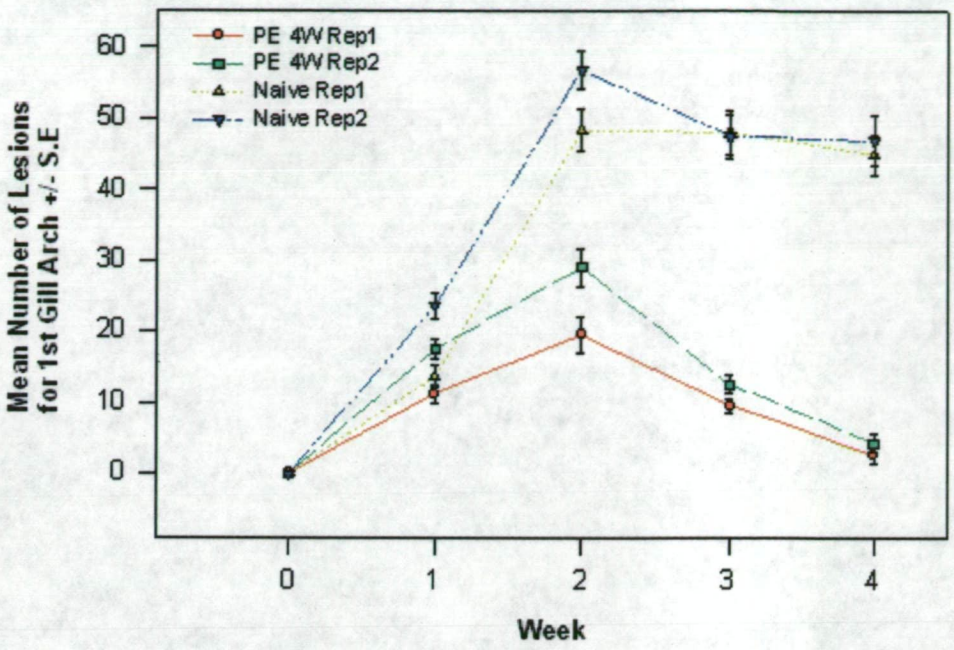
A phagocytosis assay was performed using 16 fish that were infected with AGD and 16 fish that had been acclimatized to seawater but naive in respect to AGD. The materials and methodology for the *in vivo* assay that was used here has been previously described in Chapter 2.

RESULTS

AGD challenge trial

The AGD lesion patterns for the first trial are shown in Figure 4. It is clear that the salmon that had been previously infected with AGD and allowed to recover for four weeks in freshwater were significantly more resistant to disease (F_{calc}=51.73, d.f.=1, $p<0.0001$) than the naïve fish.

Figure 4 AGD lesion patterns in fish that have previously exposed to AGD or were naïve in relation to AGD



PE 4W = Fish that had been previously infected with AGD, placed in freshwater for four weeks before being re-exposed in this trial; Naïve = Fish that were naive in relation to AGD prior to being infected in this trial.

At the first weekly examination, Tukey's grouping did not differentiate between treatment groups. However, thereafter and until the completion of the trial, Tukey's groupings distinguished the naïve and previously exposed fish; the naïve fish displayed significantly more lesions than the fish that had been previously exposed, and were obviously distressed. The mean number of lesions in the previously exposed fish declined to a low by week four, and indeed, most fish completely recovered or were displaying very few minor lesions. Out of a total number of fish included in both replicates, 20% (7 out of 40 fish in one tank and 9 out of 40 fish in the other) did not recover- that is they showed little or no change in severity or number of lesions or had died. These fish accounted for the majority of the total count of mucus patches for this group.

At the end of this challenge there were also marked differences in the mortalities that had occurred in each group of fish. Of the 40 donor fish that were used in this experiment only 30% remained alive. There were also a relatively high number of mortalities in the naïve group of fish, with 30% dying before the end of the trial. In the previously exposed group of fish, only 9% died as a result of AGD infection.

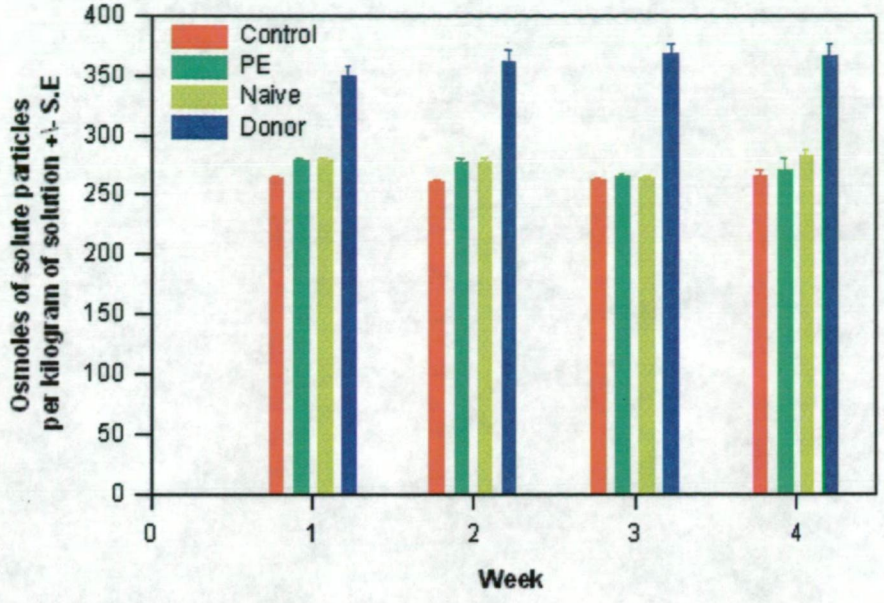
Osmolality

There was a significant difference in plasma osmolality between donor fish, infected and naïve fish and control fish ($F=263.7$, d.f. $=3$, $p<0.0001$). As can be seen in Figure 5, fish that had been chronically infected with AGD for a long period of time (donors) displayed a significantly increased plasma osmolality (as indicated by Tukey's groupings) when compared to all other groups. The Tukey's groupings did not distinguish plasma osmolality levels between fish that had been infected for the second time (previously exposed) and those being exposed for the first time (naïve). However, as previously stated both these groups of fish displayed significantly increased plasma osmolality when compared to control fish.

ELISA

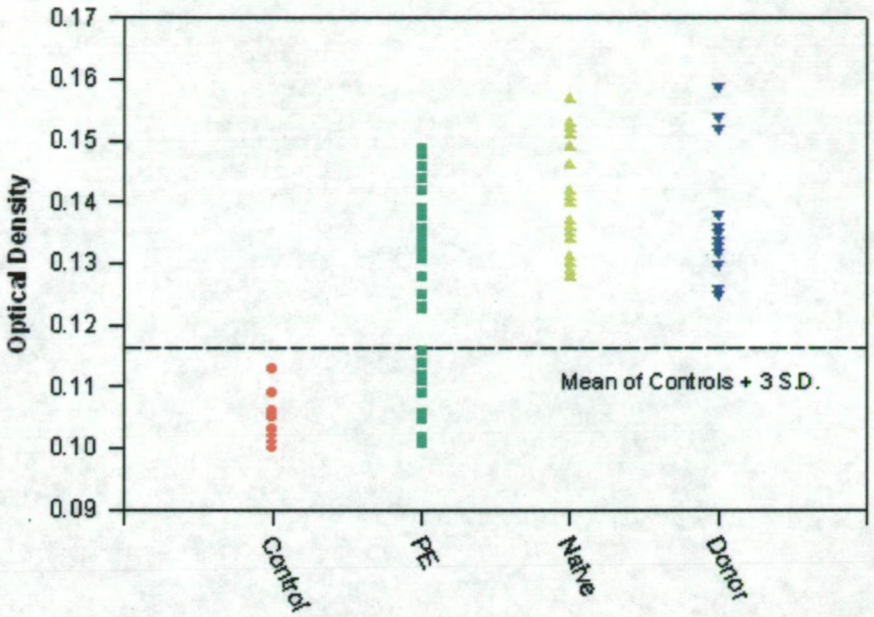
As described above, at the completion of this trial, blood and mucus samples were collected from surviving fish and ELISAs were performed using known positive and negative sera as controls. Using the convention that the negative: positive threshold is calculated as the mean of the controls plus three standard deviations of the optical density (Johnson *et al.*, 1988), it was found that there were no significant ELISA reactions with the mucus samples. However, 100% of the serum samples taken from donor and recently infected naïve fish contained anti-*Paramoeba* antibodies, as did 68% of the serum samples taken from previously infected and re-exposed fish. None of the serum samples taken from control fish contained anti-*Paramoeba* antibodies (Figure 6).

Figure 5 Serum osmolality of groups of fish with varying exposure to AGD



Control = Fish that were maintained in full salinity seawater but were not exposed to AGD; PE = Fish that were previously infected with AGD, treated with a freshwater bath and reexposed; Naïve = Fish that were previously naïve in relation to AGD but were exposed in this trial; Donor = Fish that were maintained with AGD for extended periods of time.

Figure 6 ELISA results indicating the presence/absence of anti-*Paramoeba* antibodies in serum samples taken from fish with varying exposure to AGD



Control = Fish that were maintained in full salinity seawater but were not exposed to AGD; PE = Fish that were previously infected with AGD, treated with a freshwater bath and reexposed; Naïve = Fish that were previously naïve in relation to AGD but were exposed in this trial; Donor = Fish that were maintained with AGD for extended periods of time.

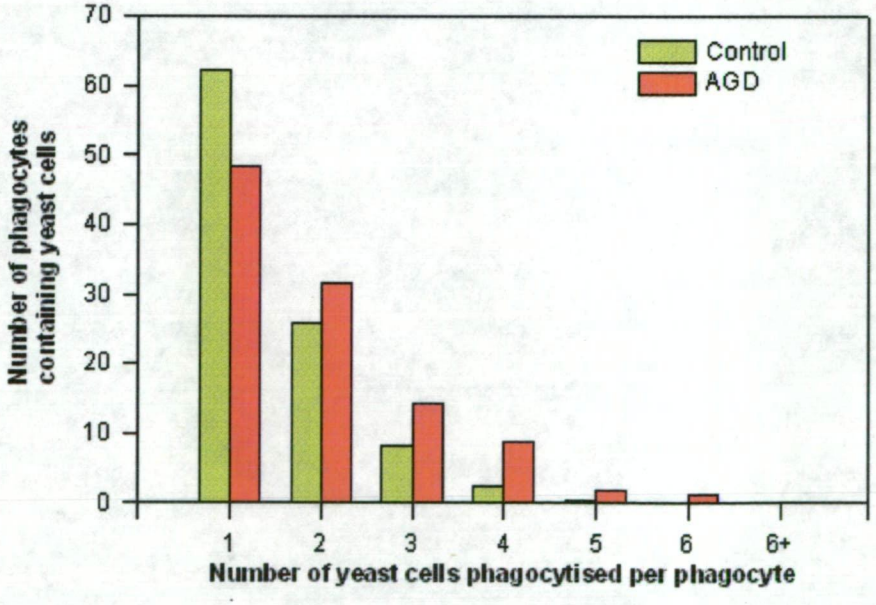
Phagocytosis assay

Head kidney cells that had been collected from the fish that had AGD demonstrated enhanced phagocytic abilities for all measured parameters. There were significant increases in the phagocytic index (PI), phagocytic capacity (PC) and phagocytic activity (PA) for AGD affected fish (Table 2). As can be seen in (Figure 7) for AGD affected fish there is a right shift in the phagocytic profile of yeast consumption per phagocyte. This represents an increased ability to engulf particles and is indicative of a stimulated nonspecific defence system.

Table 2 Comparison of phagocytic parameters in AGD infected and uninfected Atlantic salmon

Group	Phagocytic Index (PI)	Phagocytic Capacity (PC)		Phagocytic Activity (PA)
		1-3 yeast/cell	4-6+ yeast/cell	
AGD infected	1.9675	89%	11%	43.3%
Naive	1.5163	97.13%	2.87%	33.9%
Statistical analysis	F_{calc}=65.40	F_{calc}=24.77		F_{calc}=114.33
	d.f. =1	d.f. =1		d.f. =1
	p<0.0001	p<0.0001		p<0.0001

Figure 7 Comparative phagocytic assay result for fish exposed and not exposed to AGD



Control = phagocytes harvested from fish maintained in sea water but not exposed to AGD; AGD = phagocytes harvested from fish maintained in sea water and infected with AGD

DISCUSSION

This trial confirms the hypothesis that salmonids, which have suffered an attack of AGD and have been allowed to fully recover, develop significant resistance to re-infection. An interesting observation was that the previously-exposed fish initially appeared to be as susceptible as the naïve fish, but at two weeks after re-exposure, they displayed resistance. The mechanism involved is unclear but may relate to the time required for the various nonspecific defence responses to have a significant effect on the *Paramoeba*. As discussed below there is no evidence that anti-*Paramoeba* antibodies are protective.

These results, as well as those reported by Akhlaghi (1994), Howard & Carson (1994) and Zilberg & Munday (2000b), provide no indication that the detected antibodies are at all protective. There is no correlation between the presence of antibodies and a demonstrable resistance to *Paramoeba* sp. All serum samples taken from infected naïve and donor fish contained anti-*Paramoeba* antibodies, yet it was these groups of fish that displayed the most severe lesions and the highest number of mortalities. In the previously exposed fish, only 68% were positive for anti-*Paramoeba* antibodies, yet the majority of fish in this group fully recovered without further treatment.

It appears likely that the antibodies detected in the present study are relatively short lived. Once the infection is cured, as for the previously exposed group of fish (ie the stimuli to maintain the antibodies is removed), the antibodies disappear soon after. The rationale for this is that all donor fish and naïve fish that were displaying lesions were 100% positive for anti-*Paramoeba* antibodies but only 68% of the previously exposed fish, that were displaying no or few lesions were positive for anti-*Paramoeba* antibodies.

Although it is a possibility that the ELISA used in this study failed to detect the protective antibody, it is well known that all antibodies are not protective. Unless antibodies can neutralize the components of pathogenicity they are valueless in giving protection. This scenario is particularly well known for *Aeromonas salmonicida* (Cipriano & Heartwell, 1986) and *Edwardsiella ictaluri* (Saeed & Plumb, 1986). In these instances there is little correlation between detected circulating antibody titres and protective immunity. It is important to realize then that assays of antibody levels may give no indication of the degree of protection unless they are against protective antigens.

The absence of demonstrable anti-*Paramoeba* antibodies in gill mucus suggests that surface antibodies against *Paramoeba* sp. are not involved in natural immunity to

AGD. Previous studies by workers including Lumsden *et al.* (1994); Lumsden *et al.* (1993); Rombout, Taverne, van de Kamp & Taverne- Thiele (1993) and Lobb (1987) have demonstrated the existence of a mucosal antibody response in teleosts. Many of the results suggest differences in the composition of serum and mucus immunoglobulins indicating that immunoassays capable of detecting serum antibodies may not necessarily detect mucosal antibodies. However, studies by St. Louis-Cormier, Osterland & Anderson (1984) and Itami, Takahashi, Okamoto & Kubono (1988) using different teleost species did not reveal any differences between mucus and serum immunoglobulin.

Lumsden *et al.* (1995) suggest that the most likely reason experiments fail to detect a given mucosal response is due to the relative insensitivity of the detection techniques, although those authors recognize that in some cases even the most sensitive tests do not detect a response. It may be that the differences in composition of mucus and serum antibodies are dependant on the origin of the same (ie where mucosal antibodies are due to serum transudation or active transport they are most likely to have a similar composition to those detected in the serum, but where they originate from local production they are more likely to be structurally distinct). If the latter is the case for the response of Atlantic salmon to amoebic gill disease then the monoclonal antibody used in the present study would not necessarily detect a gill mucus antibody and this aspect therefore remains unresolved.

It has been demonstrated in a number of cases that soluble antigens are poor immunogens, at least for the stimulation of specific mucosal antibodies in fish. Rombout *et al.* (1989) could not identify any specific cutaneous antibodies in carp after oral administration of the antigen ferritin and Burgess (1988 as cited in Jenkins, Wrathmell, Harris & Pulsford, 1994) could only detect very low levels of antibodies in the bile and mucus in rainbow trout after immunization with human gamma globulin. Further, Bruce & Ferguson (1986) suggested that the absorption of soluble protein antigens across mucosal tracts may result in the revelation of epitopes of the molecule that are stimulatory for T-suppressor cells and also in the corresponding loss of determinants specific for T-helper cells, both of which can result in minimal immune responses. However, antibodies have been generated in the cutaneous mucus of channel catfish (Lobb, 1987), rainbow trout (Davidson, 1991, as cited in Jenkins *et al.*, 1994) and sheepshead (Lobb & Clem, 1981) in all cases with particulate antigens.

Another explanation for the apparent inability to detect a protective antibody is that these may be formed against extracellular or metabolic products of the *Paramoeba*. If this were the case, the protocol used in this study would not detect them because washed *Paramoeba* cells were used. This may also be the reason why the studies by

Akhlaghi (1994), Howard & Carson (1994) and Zilberg & Munday (2000b) have failed to detect or induce protective antibodies.

There is some evidence however, that extracellular products, including proteases are poor immunogens in salmonids. The findings of Ellis, Stapleton & Hastings (1988) did not support the assumptions by other workers, that protection afforded by vaccines composed of either crude extracellular products (ECP) or partially purified protease was the result of a specific immune response, at least in respect of antibody production. Also Hastings & Ellis (1988) showed that when rabbits were immunized using formalin inactivated toxoid of ECP of *Aeromonas salmonicida*, antibodies to at least 14 components of the ECP could be detected. In trout, antibodies to only four ECP antigens could be detected. These results indicate that extracellular antigens may require modification in order to improve their immunogenicity in fish.

Furthermore, even though fluid-phase endocytosis is recognized as the process by which antigens contained within external substances are internalized and subsequently transported to their intracellular destination, there is evidence that in fish macrophages this may not be of such importance for uptake of extracellular macromolecules as it is for turnover of cellular membranes. Lauve & Dannevig (1993) demonstrated that salmonid head-kidney macrophages endocytosed a fluid volume corresponding to approximately 1.4% of their cell volume per hour but in the same period internalised approximately 70% of its surface area. They concluded that internalisation of cellular membranes was more important but that it would be of interest to know whether the process of endocytosis may be influenced by macrophage stimulators which cause increased secretory activity of macrophages.

Even if, as the evidence is pointing, the composition of *Paramoeba* antigens is not suitable to induce a specific protective immune response, there is no reason to believe that other defence systems (ie the nonspecific) are not engaged. It is likely, and there is ample evidence that a variety of antigens elicit an innate, nonspecific defence response. *Yersinia ruckeri* antigens were found to stimulate the nonspecific cellular defence system of rainbow trout (Siwicki, Anderson & Dixon 1992). The addition of lysates from *Goussia carpelli* resulted in an increase of phagocyte activity in cells isolated from carp (Steinhagen & Hespe, 1997). *Aeromonas salmonicida* infection was shown to increase the levels of lysozyme, protease activity, haemolytic activity as well as serum complement (Moyner, Roed, Sevatdal & Heum, 1993) and carp infected with *Eimeria subepithelialis* displayed augmented serum lysozyme levels and phagocytic ability (Studnicka & Siwicki, 1990).

The augmentation of phagocytic ability demonstrated in this study may have been initiated by soluble antigens present in a layer of extracellular material that is,

presumably, laid down as the *Paramoeba* moves along. Pinto da Silva & Martinez-Palomo (1974) demonstrated that in actively moving *Entamoeba histolytica* cells a continuous shedding of surface components appears to take place. The thin layer of material that accumulates as microexudates has cytochemical properties similar to those of the antigenic surface coat. Given the evidence above, it is possible that these soluble antigens elicit stimulation of the nonspecific defence system of fish affected by AGD. The remote siting of the *Paramoeba* in relation to the specific humoral immune system (ie *Paramoeba* are mainly superficial and have never been noted to be phagocytosed) provides further evidence for such a mechanism.

On the evidence thus far, including the absence of demonstrable anti-*Paramoeba* antibodies in the mucus and demonstrated lack of protection by serum antibodies, together with the remote siting of AGD infection in respect to the humoral immune system one must begin to think about the role of the nonspecific defence system in resistance to AGD. Indeed, the phagocytic assay results indicate that AGD infection may lead to the immunostimulation of the nonspecific defence system. Whilst the process of phagocytosis itself is unlikely to play a large part in resistance to AGD, augmentation of phagocytic ability is indicative of possible increases in numerous other nonspecific defence parameters. These factors are clearly of interest, especially where there is a coincidental increase in disease resistance.

It is important to keep in mind that because of the complex interrelations between the nonspecific defence system and the specific immune system as discussed in Chapter one, it seems possible, if not likely, that stimulation of the nonspecific defence system may result in stimulation of the specific immune system (ie an increase in the speed and magnitude of a specific immune response). This will be discussed in more detail in later chapters.

There are obviously deleterious effects of AGD infections and Munday *et al.* (1990) have previously reported that clinically affected fish have markedly elevated blood sodium levels and subclinically affected animals have lower, but still abnormal levels. The results of the present osmolality experiment confirm previous results that fish with long-established lesions suffer from osmoregulatory failure. However, while fish exhibiting extensive, but short term lesions exhibit elevated plasma osmolality, the recorded levels do not indicate osmoregulatory failure.

It is probable that the decreased number of chloride cells observed in the affected areas of clinically affected fish (Munday *et al.*, 1990) inhibit osmoregulation. The osmoregulatory failure that is seen in chronically affected fish is likely to be related to the formation of gill 'cysts'. In a proper functioning gill, chloride cells open to the surface of the gill and are in constant contact with the surrounding environment.

When these cells form 'cysts' in the gills and are no longer in contact with the environment of the fish, the massive flux of salt across the gills can no longer occur. This results in increasing osmotic imbalance and without treatment, often death.

In affected fish that are freshwater-bathed, the immediate improvement in general health can be explained by the reduced need for the fish to pump salt out of their system against an ionic gradient and a partial restoration of ionic balance. Work conducted by Bakke, Bjerknes & Oevreeide (1991) confirms that Atlantic salmon held in freshwater for varying periods of time show significantly lowered sodium and chloride values when compared to fish held in saltwater. These workers reported that although plasma sodium levels return to pretreatment levels quickly after return to saltwater, chloride levels show a somewhat slower rise back to pretreatment levels. Although these workers did not further elaborate this result, it may be partly explained by the fact that sodium ions leave the gill epithelium passively via the leaky tight junctions between chloride cells as opposed to chloride secretion which is regulated by the active ATPase sodium pump.

The longer term recovery that is seen in AGD-affected freshwater bathed fish is likely to result from a significant reduction in the numbers of amoebae, which in turn, would allow for normalisation of the gill epithelium and restoration of functional osmoregulation.

A particularly interesting result from this trial was the group of previously exposed fish that did not develop a useful resistance to reinfection with *Paramoeba* sp. suggesting that these fish may be immunological subperformers. Given that a number of the freshwater baths carried out during the high-risk season are due to a group of fish apparently relapsing, while the remaining fish appear resistant, it was of considerable importance that this issue was further considered. If the number of baths could be reduced, this would be of significant value to industry.

The series of experiments in the next chapter therefore deals with, *inter alia*, a treatment for this group of sub-performers.

CHAPTER 5

THE EFFECT OF LEVAMISOLE ON THE NONSPECIFIC DEFENCE SYSTEM OF ATLANTIC SALMON

Contents

INTRODUCTION

Nonspecific defence mechanisms are important to all vertebrates, and fish, especially cold-water species, depend more heavily on these nonspecific mechanisms than do mammals (Avtalion, 1981). Furthermore, in those instances where pathogens, such as *Paramoeba* sp., infect sites that are relatively insulated from the specific humoral immune system, such as mucous surfaces, there seems to be few available options for treatment or prophylaxis, other than direct chemical therapeutics, or modulation of the nonspecific defence system. As a result of these considerations, and also because there was a group of fish which did not appear to develop resistance to AGD after freshwater bathing it was decided to investigate the use of immunomodulators.

In the last decade there has been increasing interest in the modulation of the nonspecific defence system of fish for both treatment and prophylaxis against disease. A number of substances, including levamisole, have been shown to heighten the nonspecific defence system capacity. Studies including those by Jeney, Galeotii & Volpatti (1994), Mulero, Estaban, Munoz & Meseguer (1998) and Symoens & Rosenthal (1977) indicate that treatment with levamisole leads to an enhanced state of resistance to various kinds of infections. Levamisole is especially known for its ability to restore immunological function in immunocompromised individuals.

In this series of experiments, modulation of the nonspecific defence system including lysozyme, reactive oxygen species, phagocytic ability and lymphocyte stimulation in Atlantic salmon following levamisole treatment was investigated. This is the first record of the use and efficacy of levamisole on the nonspecific defence system of Atlantic salmon.

Levamisole is a levo-isomer of tetramisole. The chemical name of this synthetic compound is 1-2, 3, 5, 6-tetrahydro-6-phenylimidazo[2,1-*b*] thiazole (Arundel, 1995). The compound was originally registered as a broad spectrum anthelmintic in ruminants and has since been used extensively and safely in veterinary and human medicine (Anon., 1991; Arundel, 1995). Levamisole is a known T cell stimulator and immunomodulator in mammals.

Levamisole is rapidly adsorbed and excreted. Peak blood levels in farm animals are reached two to three hours following oral dosage and concentrations then decrease with little being present after 20 hours. In farm animals it is almost totally excreted in the urine and bronchial mucus. In man levamisole has a plasma half-life of about four hours, is extensively metabolized in the liver and is virtually eliminated from the body within two days (Symoens & Rosenthal, 1977). The half life in eels at 19-23°C is remarkably similar at about four hours (Blanc, Loussouarn & Pinault, 1991).

Levamisole is quite stable in acid aqueous media but hydrolyzes in alkaline solution (Symoens & Rosenthal, 1977). In relation to levamisole being used as a treatment for AGD, this means that in seawater, treatment would be ineffective unless the pH is adjusted and the pH of the freshwater treatment should be measured before levamisole is added. The benefit of levamisole hydrolysing in an alkaline media is that the treatment bath may be released with minimal effect on the environment.

Levamisole has been demonstrated to augment both the specific immune and nonspecific defence systems of fish, but as has been documented in previous chapters, there is no correlation between detectable antibody against the *Paramoeba* sp. causing AGD and resistance to the disease. Therefore it was argued that there

would be little benefit given these circumstances in considering responses of the specific (adaptive) immune system in preference to those of the nonspecific defence system.

The functional abilities monitored in this study such as phagocytic function, reactive oxygen species production, lymphocyte proliferation, and lysozyme are important elements of the nonspecific defence system. However, their contribution to and significance in Atlantic salmon defence mechanisms are dependent on the characteristics of the disease agent they act upon. This aspect of immunomodulation will be dealt with in a later chapter.

Given that it had been previously demonstrated that fish infected with *Paramoeba* sp. displayed heightened phagocytic ability and appeared to lack protective antibodies, it was hypothesised that if a substance could be found that achieved immunostimulation of the nonspecific defence system of Atlantic salmon then protection against *Paramoeba* sp. may be given to these fish. Levamisole was the immunomodulator of choice because it had been shown to stimulate the nonspecific defence parameters in other fish species and it was also known for its ability to treat immunocompromised animals (ie the group of Atlantic salmon that do not display resistance to re-infection with AGD).

MATERIALS AND METHODS

Treatment of Fish

All experimental fish were maintained as described previously in Chapter 2. Of the fish in each group, half were bathed in a freshwater bath with levamisole added at a rate to give 5mg.l^{-1} active principle in the water (treatment group) and the other half in a plain freshwater bath (control group). Fish were killed 14 days post-treatment.

Immunoassays

Lysozyme Assay

A turbidometric assay utilizing *Micrococcus lysodeikticus* lyophilised cells (Sigma) was used to determine lysozyme concentrations in the serum and mucus removed from Atlantic salmon as described in Chapter 4. Eight samples were used in each assay. The assay method was a modification of that used by Sankaran & Gurnani (1972) who reported differences in the optimal pH and buffer molarities according to whether the fish were from freshwater or seawater. Thus, a series of assays was

conducted with pooled mucus and serum samples to optimise the test for use with Atlantic salmon maintained in seawater. While it was recognised that different buffers may further optimise results it was important to maintain some comparability so the same buffer (0.04M phosphate buffer) was use for all assays.

M. lysodeikticus was suspended in phosphate buffer at a concentration of 0.25 mg.ml^{-1}. Two hundred microlitres of serum or mucus, diluted with an equal volume of PBS, were added to 1.3ml of the substrate solution at 25°C and the optical density at 450ηm read immediately. After 30 minutes incubation in a humidified environment at 25°C the optical density was measured once again. Lyophilised hen egg white lysozyme (HEWL) was used to develop a standard curve.

Serum and mucus lysozyme values are expressed as μg.ml^{-1} equivalent of hen egg white activity and were derived using the equation derived for the line of best fit.

Phagocytosis Assay

The method used for the phagocytosis assay has been previously described in Chapter 2.

Two groups of fish were used in this assay; the first group was treated with levamisole and the second acted as a control group and were bathed in freshwater only. There were 20 fish per group.

Reactive Oxygen Intermediate - Superoxide Anion Assay.

Head kidney cells used in this assay were collected as described for the phagocytosis assay in Chapter 2. The only difference being that the white blood cell band was removed and washed twice with 3ml of supplemented L15 medium. After the final wash, the supernatant is removed, the pelleted cells resuspended in 3ml of supplemented FPS, and the cell number and viability determined by hemocytomter counting and trypan blue exclusion, respectively.

Superoxide produced by the macrophages isolated from the head kidney was measured by the reduction of ferricytochrome C as described by Secombes, Chung & Jefferies (1988) and Zelikoff, Wang, Islam, Twerdok, Curry, Beaman & Flescher (1996). The specificity of the reaction was demonstrated by preventing the reduction of ferricytochrome C with exogenous superoxide dismutase (SOD) which dismutates any O_2^- generated to hydrogen peroxide.

The amount of O_2^- produced in respiratory burst was quantified by comparing cells taken from an individual fish using four different reaction mixtures. Each of these

four mixtures contained 500µl of ferricytochrome C (Sigma) (final concentration of 2mg.ml^{-1} prepared in supplemented fish physiological saline) to which had been added 10^6 kidney cells (in a total volume of 250µl of supplemented Leibovitz medium). The first two reaction mixtures measured basal levels of O_2^- so no membrane stimulant was added to these mixtures. Exogenous SOD (125 µL at a final concentration of 37.5 µg.ml^{-1} prepared in Hanks buffered salt solution (HBBS)) was added to one of these mixtures so any basal level O_2^- was inhibited. The second two reaction mixtures mimicked the first, with the addition of 100µl of the soluble stimulant phorbal myristate acetate (PMA) (Sigma) (at a final concentration of 2.0µg.ml^{-1}, prepared in dimethylsuphoxide, working solution of 100µg.ml^{-1} prepared in HBSS). An additional tube that contained all of the above-mentioned reagents, but without cells, acted as the reaction blank. Fish physiological saline was added to all the mixtures to bring the final volume up to 1ml.

Each of the mixtures was vortexed for 30 seconds before 200µl aliquots were placed in triplicate in 96-well microtitre plates. The absorbance was measured at 550ηm every ten minutes for two hours and again at 24 hours. Between readings the plates were placed in a humidified incubator at 30°C.

Change in absorbance was calculated by first subtracting the mean of the blank wells from all other wells and then subtracting the absorbance of the wells containing SOD from that of the non-SOD-containing wells. The results were expressed by converting the optical density readings to ηmol O_2^- per 10^5 cells by multiplying with the correction factor of 15.87 as given by Pick & Mizel (1981).

Mitogen-induced lymphocyte proliferation

Lymphocytes in the peripheral blood and the spleen were isolated from each of ten fish that had been treated with either a levamisole and freshwater bath or a freshwater only bath.

Lymphocytes were isolated by buoyant density gradient centrifugation. This method is known to result in high recovery (>95%) of lymphocytes (Braun-Nesje *et al.*, 1981). For determination of the lymphocyte percentage in each suspension, slides were stained with Wright's stain and examined morphologically using light microscopy. The final percentage of lymphocytes averaged 96.2%. The remainder of the cell suspension consisted of mostly macrophages but at times contained thrombocytes and/or granulocytes.

Peripheral blood lymphocytes were obtained by caudal vein puncture with a 10ml sterile syringe preloaded with heparin 100µg.ml^{-1} in PBS (Sigma) fitted with a 21

gauge needle. The collected blood was diluted in culture medium by 1:2 to 1:3 and centrifuged at 60*g* for 15 minutes in 10ml tubes. The supernatant was layered on 2ml of Ficoll (Nycomed Pharma AS, Norway) and the tubes were centrifuged at 60*g* for five minutes and then at 550*g* for 30 minutes. The interface containing the lymphocytes was removed and washed twice in PBS +2% foetal calf serum (FCS). Cell viability (≥95%) was confirmed using trypan blue exclusion (0.25% trypan blue solution).

To obtain spleen lymphocytes, the fish were killed and the spleen was dissected out. The spleen was placed in a sterile petri dish and flushed by injecting culture medium at various points of the organ. Flushing was performed ten times, using the same 4ml of culture medium. This lymphocyte suspension was then layered on 2ml of Ficoll and treated as above.

The mitogens, phytohaematoagglutinin (PHA) (Sigma), concanavalin A (ConA) (Sigma), pokeween mitogen (PWM) (Sigma) and lipopolysaccharide from *E. coli* (LPS) were reconstituted in RPMI-1640 (Sigma), aliquoted and stored at –20°C. Spleen or peripheral blood lymphocytes were added to 96-well plates (Corning glass works, New York) in 100µl aliquots ($2x10^5$ lymphocytes per well). Cultures were adjusted to 200µl with RPMI-1640 for the unstimulated controls or the mitogen diluted in RPMI-1640.

The optimal response for the respective various mitogen concentrations was determined by conducting pre-tests using the method described below. The final concentrations of mitogen for which the results are reported here were $20\mu g.ml^{-1}$ for PHA and $150\mu g.ml^{-1}$ for LPS. The responses to all concentrations of ConA and PWM tested were so weak that these mitogens were left out of the final experiment.

The lymphocytes were cultured in a Gallencamp-Incubator (UK) in water saturated air at 15°C for 48 hours. Each well was then pulsed with 0.5 µCi ^{3}H-Thymedine (Amersham, USA) and after a further 24 hours the cells were harvested onto harvester filters (Titertek) using a cell harvester (Skarton combi cell harvester, USA). After drying, filter pellets were passed into counting tubes and 1.5ml of scintillation liquid (Amersham) was added. Incorporated radioactivity (^{3}H-Thymedine in newly synthesised DNA of proliferating cells) was assessed using a scintillation counter (Casey/LKB- Wallace).

Modulation can be indicated using background counts and/or the stimulation index (SI). The SI is calculated by dividing the counts per minute (CPM) of the stimulated culture by the background CPM.

Haematocrit and leucocrit

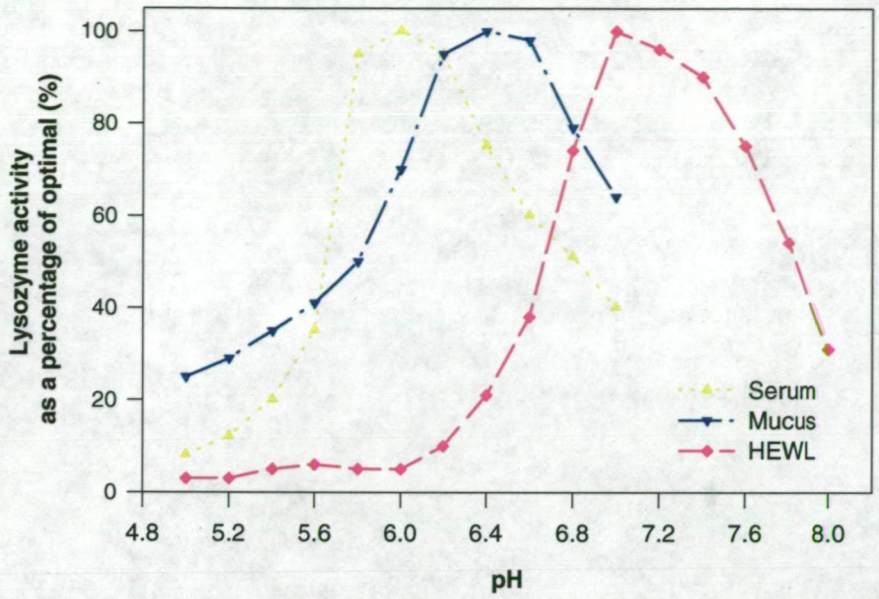
Twenty fish from each of the levamisole and freshwater treated group and the freshwater only treated group were anaesthetised as previously described and a blood sample taken from the caudal vessel. Heparinised haematocrit capillary tubes (Chase Instrument Corporation, Glens Falls, NY) were filled to the red line. Duplicate samples were taken from each fish. The tubes were then centrifuged at 400*g* for one minute using a haematocrit centrifuge. The percentage erythrocyte (haematocrit) and leukocyte (leucocrit) volume was calculated by overlaying the tubes on a sliding scale haematocrit reader.

RESULTS

Lysozyme assay

The lysozyme activity in Atlantic salmon serum was found to have an optimum at pH 5.8 (Figure 8). Lysozyme activity of mucus was also pH dependant with an optimum of pH 6.2. HEWL in contrast, exhibited an optimal activity at pH 7.0 with sharp declines in activity at acidic and alkaline pH. While it is acknowledged, that because of the above results, HEWL may not be the best standard to use for lysozyme assays involving fish, it is readily available and consistently used by workers in the field. It was used in this study to maintain some comparability between results of our studies and those reported in the literature.

Figure 8 Effect of pH on the lysozyme activity of serum, mucus and hen egg white lysozyme



The standard curve developed using HEWL is shown in Figure 9. A second order polynomial regression was fitted to the standard (r^2= 0.9862) whereby resultant optical density readings for serum and mucosal lysozyme for fish could be converted to equivalent HEWL concentration using the formula where lysozyme concentration = 0.4185 - 5.5969 x change in OD + 65.3178 x change in OD^2. In all cases serum and mucus obtained from levamisole-treated fish displayed a significantly increased lysozyme activity when compared to control fish (F_{calc} =168.2, d.f. =1, $p<0.0001$; F_{calc} =221.27, d.f. =1, $p<0.0001$, respectively) (Figure 10,Figure 11). Mucosal samples had consistently higher lysozyme activity than serum samples. Furthermore, when samples were heated-treated, thus inactivating the heat labile components of the samples, there was a consistent drop in the lytic activity of all mucus and serum samples whether they were levamisole treated or not.

Figure 9 Standard curve for hen egg white lysozyme

A second order polynomial regression was fitted to the curve (r^2 =0.9862)

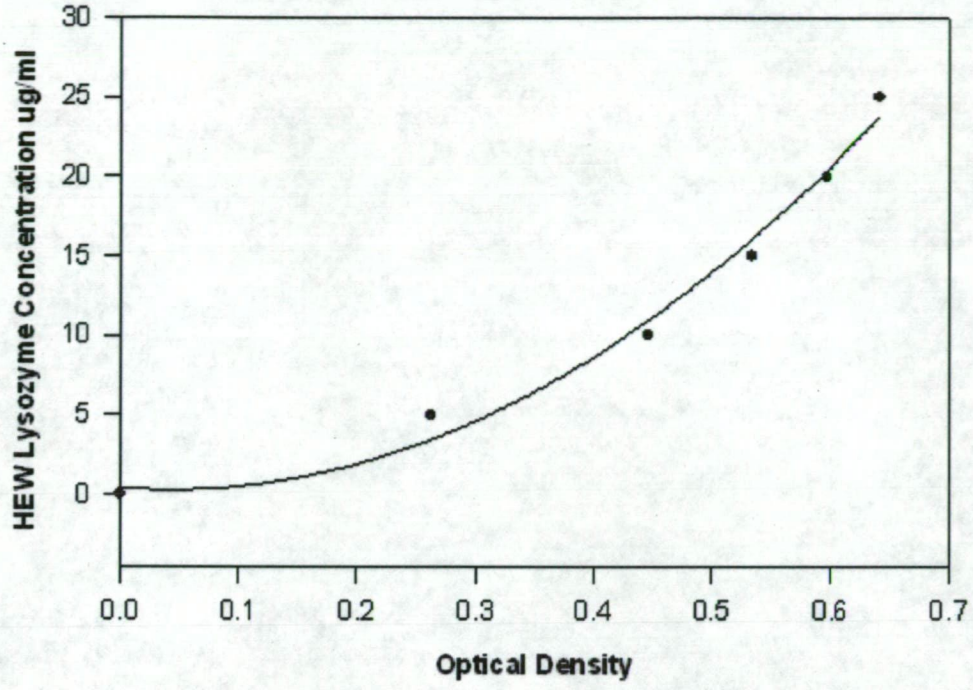
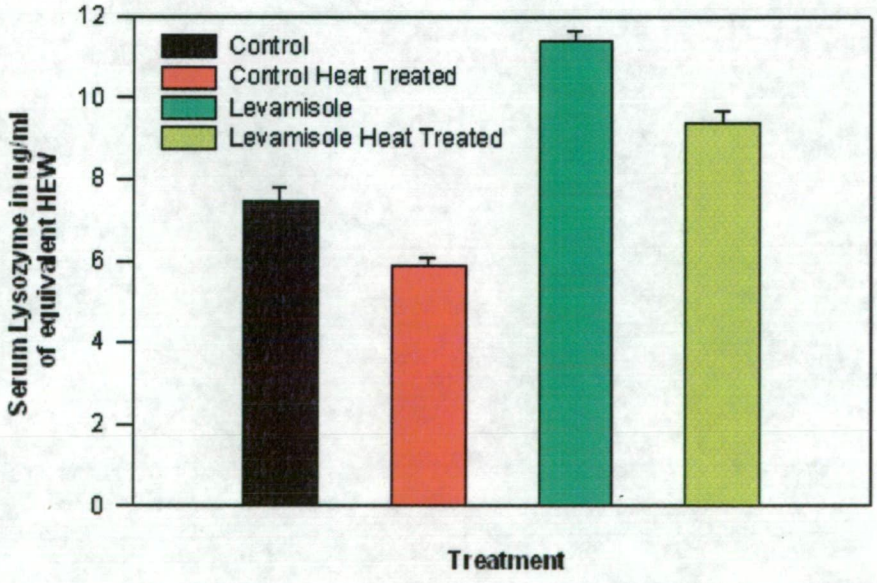
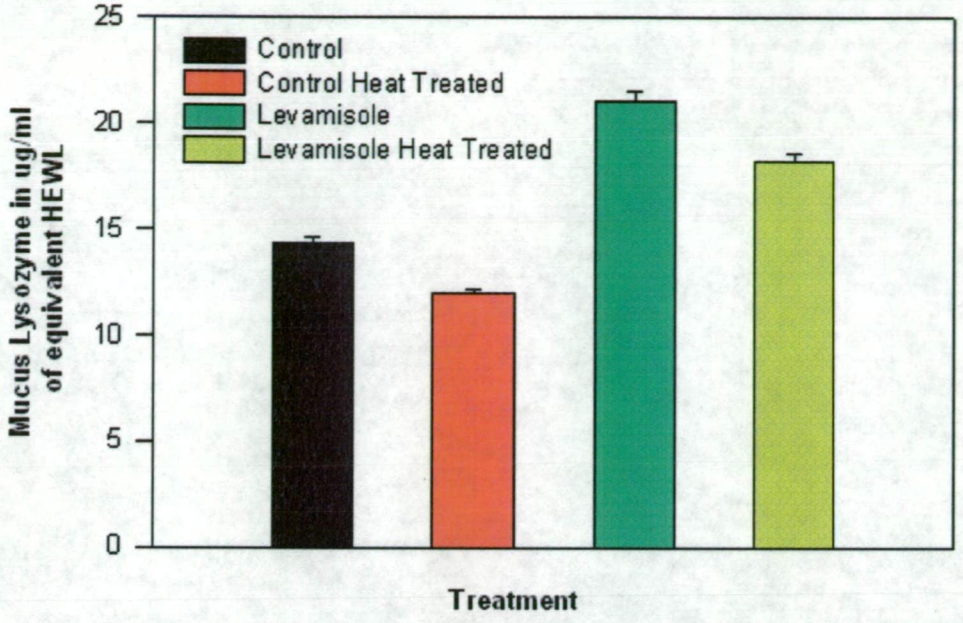


Figure 10 Effect of levamisole on serum lysozyme of Atlantic salmon



Control = Serum taken from fish that had been given only a freshwater bath; Control Heat Treated = as above but the serum was heated; Levamisole = serum taken from fish that had been treated with a freshwater and levamisole bath; Levamisole Heat Treated = as above but the serum was heated.

Figure 11 Effect of levamisole on mucus lysozyme of Atlantic salmon



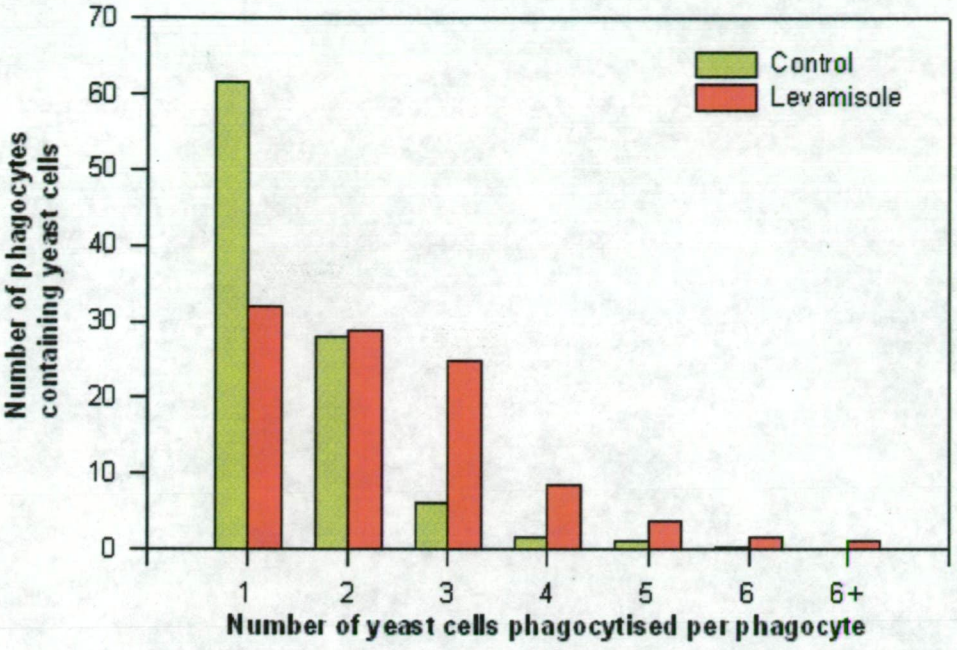
Control = Mucus taken from fish that were given a freshwater bath only; Control Heat Treated = as above but the mucus was heated; Levamisole = mucus taken from fish that had been treated with freshwater and levamisole bath; Levamisole Heat Treated = as above but the mucus was heated

Phagocytosis assay

Head kidney cells that had been harvested from fish treated with levamisole demonstrated significantly enhanced phagocytic function for all measurements of phagocytic ability (ie phagocytic index-PI, phagocytic capacity-PC and phagocytic activity-PA).

There was a significant increase in the number of yeast cells consumed per phagocyte in fish treated with levamisole (F_{calc}= 338.27, df = 1, p<0.0001). The treated fish had a PI of 2.31 compared to control fish with a PI of 1.54. As can be seen in Figure 12, for levamisole-treated fish there is a right shift in the phagocytic profile of yeast consumption per phagocyte, with a significant increase (F_{calc}= 375.02, df = 1, p< 0.0001) in PC in harvested phagocytes. Of the phagocytic cells harvested from fish that had been treated with levamisole 50.03% had consumed one or more yeast cells. This is a significant increase (F_{calc}= 61.9415, df = 1, p< 0.0001) when compared to a phagocytic index of 33.47% for control fish.

Figure 12 Comparative phagocytic assay result for fish treated or not treated with levamisole

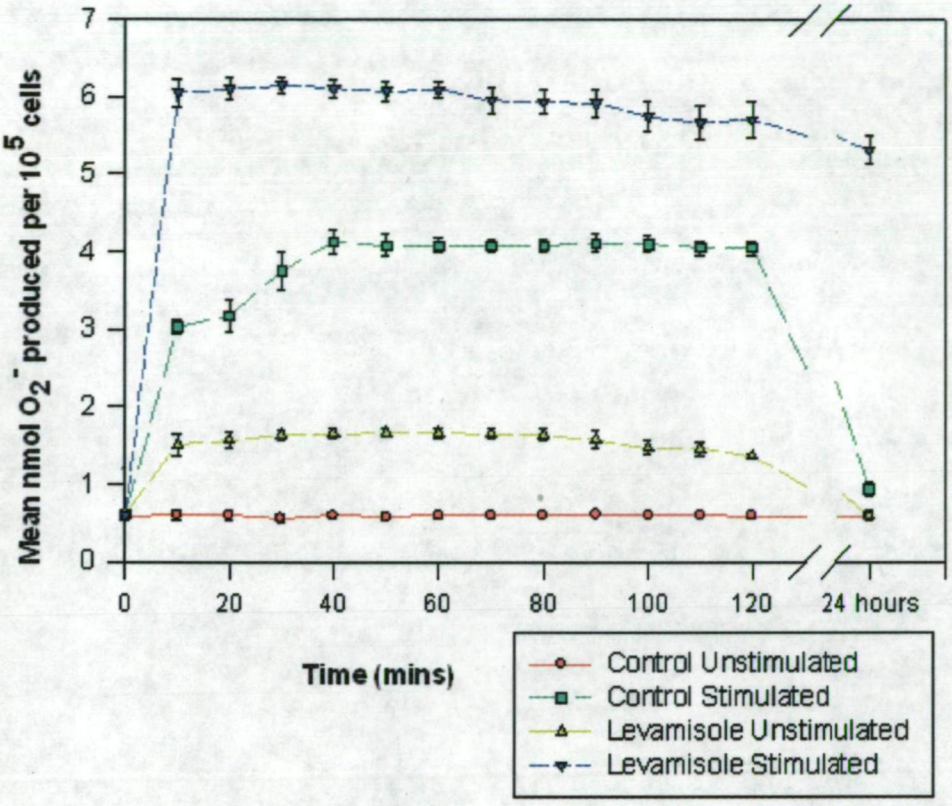


Control =Phagocytes taken from fish given only a freshwater bath; Levamisole =Phagocytes taken from fish treated with a freshwater and levamisole bath.

Superoxide anion production

In all experiments the addition of exogenous SOD inhibited the reduction of ferricytochrome C by the macrophages, confirming that the assay was specific for O_2^-. Figure 13 shows that the macrophages from levamisole-treated fish had a significantly increased O_2^- production with or without PMA stimulation (F_{calc}=209.66, d.f.=3, $p<0.0001$). The Tukey's test distinguished between all treatments. Furthermore, at 24 hours post-harvest the production of O_2^- in PMA stimulated macrophages from levamisole treated fish remained at elevated levels while all other groups returned to basal levels.

Figure 13 Superoxide production from macrophages of fish treated or not treated with levamisole



Control Unstimulated = macrophages harvested from fish that were given a freshwater bath only prior to harvest, but macrophages were not treated with PMA post harvest; Control Stimulated = as above, but macrophages were treated post harvest with PMA; Levamisole Unstimulated = macrophages harvested from fish that were given a freshwater and levamisole bath prior to harvest, but but macrophages were not treated with PMA post harvest; Levamisole Stimulated = as above, but macrophages were treated with PMA post harvest.

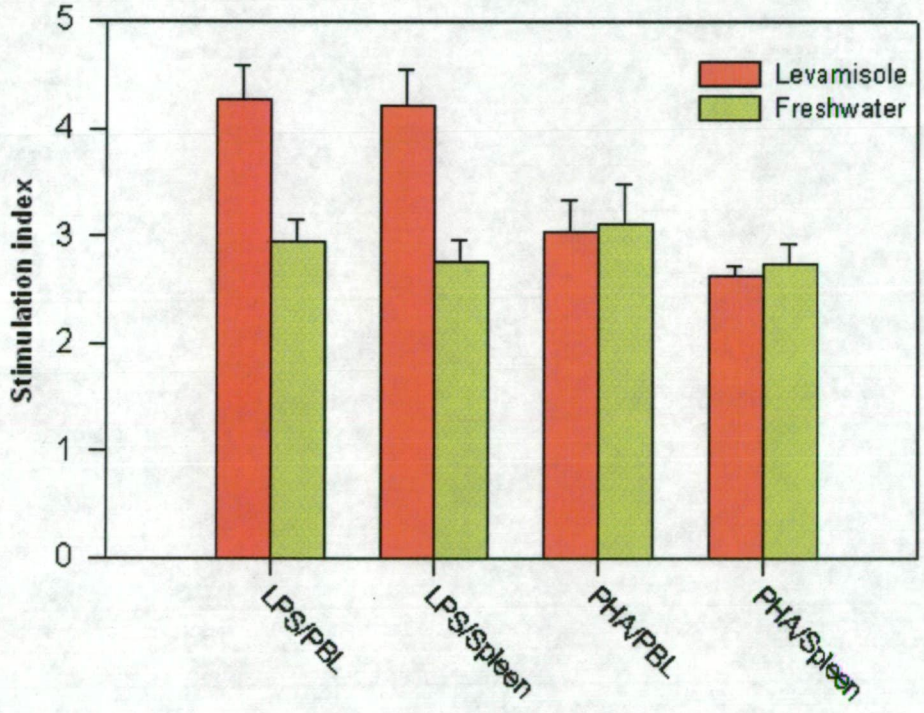
Mitogen-induced lymphocyte proliferation

It is thought the results of these lymphoproliferation assays were affected by increased cortisol levels as a result of stresses experienced by the fish being transported for three hours and subsequently being held in small tanks, prior to the lymphocytes being harvested. Even though these practical difficulties indicate that the results presented below should be interpreted with caution, trends indicating certain actions of levamisole on the lymphocyte population were obvious.

There were no significant differences between the stimulation indices for either the peripheral blood lymphoctye population or the splenic lymphocytes, whether treated with PHA or LPS (F_{calc}=0.020, d.f.=1, p=0.890; F_{calc}=0.338, d.f=1, p=0.569; F_{calc}=3.51, d.f=1, p=0.080; F_{calc}=3.72, d.f.=1, p=0.072, respectively) (Figure 14).

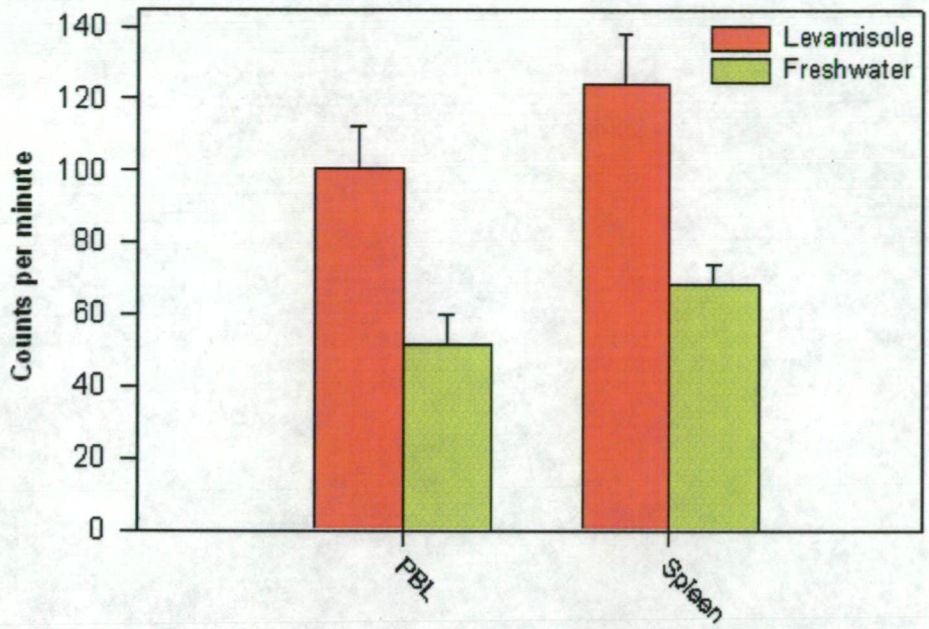
There were however significant differences in the basal levels of lymphocytes of peripheral blood and splenic origins, with the counts per minute being significantly increased for levamisole treated Atlantic salmon (F_{calc}=11.59, d.f=1, p<0.05; F_{calc}=14.09, d.f=1, p<0.05) (Figure 15).

Figure 14 Stimulation indices for lymphocytes harvested from fish treated or not treated with levamisole



Levamisole = lymphocytes taken from fish that had been treated with a freshwater and levamisole bath prior to harvest; Freshwater = lymphocytes taken from fish that had been given only a freshwater bath; LPS/PBL = Peripheral blood lymphocytes treated with LPS; LPS/Spleen = lymphocytes harvested from the spleen and treated with LPS; PHA/PBL = Peripheral blood lymphocytes treated with PHA; PHA/Spleen = lymphocytes harvested from the spleen and treated with PHA.

Figure 15 Basal level counts for lymphocytes harvested from fish treated or not treated with levamisole



Levamisole = lymphocytes harvested from fish that had been treated with a levamisole and freshwater bath prior to harvest; Freshwater = lymphocytes taken from fish that had been treated with a freshwater bath only; PBL = peripheral blood lymphocytes; Spleen = lymphocytes harvested from the spleen.

Haematocrit and leucocrit

No significant differences were found between levamisole-treated and control fish for haematocrit and leukocrit (F_{calc}= 2.71, d.f. = 1, p= 0.1078 and F_{calc}= 0.88, d.f. = 1, p= 0.3535 respectively). Treated fish exhibited a mean haematocrit level of 48.8% and a mean leucocrit level of 1.78%. Control fish had a mean haematocrit level of 46.9% with a leucocrit value of 1.78%

DISCUSSION

In this study all groups of fish given a bath treatment of levamisole had increased non-specific defence factor values when compared to control fish. Evidence for the efficacy of immunostimulants is of particular interest in circumstances where the host's capacity to mount an immune response is not sufficient to successfully combat an invasion by pathogenic microorganisms. In these circumstances, the demonstrated increases in the magnitude of the defence reaction may be adequate to ensure reasonable protection.

Levamisole has been shown to act as an immunostimulant in a number of fish species. Siwicki (1987, 1989) described the immunostimulatory activity of levamisole in carp spawners, with treated fish displaying elevated leucocyte and neutrophil numbers, enhancement of phagocytic activity, leucocyte migration and myeloperoxidase activity as well as increases of lysozyme levels and natural antibody titres. In their studies, Kajita, Sakai, Atsuta & Kobayashi (1990) showed that levamisole stimulated phagocytic activity, chemiluminescence responses and natural killer cell activity in rainbow trout. These workers also demonstrated activation of the alternative complement pathway. Levamisole also has an immunostimulatory effect on the sea bass, *Dicentrarchus labrax* and the gilthead seabream, *Sparus auratus* as shown by Jeney, Galeotti & Volpatti (1994, 1995) and Mulero, Esteban, Munoz & Meseguer (1998) respectively. Both groups demonstrated significant increases in respiratory burst and phagocytosis activities.

Granulocytes and mononuclear phagocytes or macrophages play a central role in the cellular part of the nonspecific defence of fish (Dalmo, Bogwald, Ingebrigtsen & Seljelid, 1996). In the present study three phagocytic functions were assessed and after treatment with levamisole all were increased. Whilst phagocyte killing mechanisms are not well established in fish many studies have shown that fish phagocytes have potent bactericidal and larvacidal activity and thus possess, presumably, both intracellular and extracellular killing mechanisms (Secombes & Fletcher, 1992). Further, the presence of nonspecific cytotoxic cells (NCC), similar to mammalian nautral killer (NK) cells have been characterized from various fish species including channel catfish (Evans, Graves, Cobb & Dawe, 1984) and tilapia (Faisal, Ahmed, Peters, & Cooper, 1989). In mammals these cytotoxic cells perform antimicrobial and antiparasitic functions. An antiparasitic function of these cells has also been shown in channel catfish (Evans *et al.*, 1984).

If extracellular killing can be facilitated then many implications may follow, particularly where parasites such as the causative agent of AGD infest areas that are relatively protected from the effects of antibody-mediated immunity. In this context, Whyte *et al.*, (1989) demonstrated that normal macrophages from rainbow trout are capable of killing non-opsonized diplostomes. Faisal, Perkins & Cooper (1990) suggested that the redistribution of nonspecific cytotoxicity in response to parasitic invasion, such as occurs in arrowtooth flounder, *Atheresthes stomias* infested with the copepod, *Phrixocephalus cincinnatus* (Faisal et al, 1990) and in the channel catfish, *Ictalurus punctatus*, infested with whitespot, *Ichthyophthirius multifiliis* (Graves, Evans & Dawe, 1985) may be defence mechanism by which the host attempts to counteract the pathogen during early stages of infection prior to its establishment in the host. It may be relevant that Ellis (1981) suggested that the fish

neutrophils may carry out a microbicidal role extracellularly rather than intracellularly.

Extracellular killing may also involve the release of free radicals such as superoxide anions and hydrogen peroxide through the respiratory burst and enzymes such as lysozyme.

It is well documented that the first reaction in the respiratory burst is the one electron reduction of oxygen to superoxide (O_2^-), catalysed by NADPH oxidase associated with phagocyte membrane (Drath & Karnovsky, 1975; Fihlo, Giulivi & Boveris, 1993). In the presence of the cytoplasmic enzyme superoxide dismutase (SOD), superoxide anions are then converted to hydrogen peroxide (H_2O_2). Superoxide, and to a great extent, H_2O_2 are highly reactive and toxic reactive oxygen intermediates (ROI); with H_2O_2, in conjunction with myeloperoxidase (MPO) and halide, forming the basis of a potent antibacterial system (Chung & Secombes, 1988). Other toxic ROIs can be generated, such as hydroxyl radicals (.OH) and singlet oxygen ($^1O_2$). Singly or collectively, these ROIs can participate in the cell-mediated destruction of bacteria, fungi, protozoa (Anderson, 1994, Fernandes & Assreuy, 1997) and helminths (Kazura, Fanning, Blumer & Mahmoud, 1981; Yazdanbakhsh, Tai, Spry, Gleich & Roos, 1987).

There is also evidence that activated macrophages produce large amounts of an unstable gas, nitric oxide (NO) (Moncada, Palmer & Higgs (1991). The role of this reactive nitrogen intermediate (RNI) in killing several pathogenic agents such as fungi and intracellular protozoans has been well reported (Fernandes & Assreuy, 1997). In one instance, NO has been shown to exhibit cytotoxic effects on an extracellular parasite; *Schistosoma mansoni* (James & Glaven, 1989). There is evidence that peroxynitrite, a reaction product of NO and superoxide anion may be the cytotoxic effector responsible for the killing of several agents (Fernandes & Assreuy, 1997).

All of the ROIs, and presumably the RNIs, mentioned above are apparently produced by fish phagocytes (Scott & Klesius, 1981; Chung & Secombes, 1988; Aksnes & Njaa, 1981; Secombes & Fletcher, 1992), and there is evidence that both intracellular and extracellular killing of teleost pathogens are effected by these reactive moieties (Stave, Roberson & Hetrick, 1984; Whyte *et al.*, 1989; Jorgensen, Sharp, Secombes & Robertsen, 1993). If this is the case for fish infected with *Paramoeba* sp., then even if this killing were at the expense of localised cellular health, it must be seen as an advantage given the excellent regenerative powers of fish. It is relevant that, Symoens & Rosenthal (1977) reported that levamisole treatment leads to an

enhanced state of healing and wound repair. Presumably, enhanced healing would be of some benefit to fish with disrupted gill epithelia.

While lysozyme is generally recognized as being part of the humoral nonspecific defence system it could be argued that it makes up part of the cellular nonspecific component given that it emanates from phagocytes. Lysozymes are widespread enzymes occurring in many teleost tissues and secretions (Lindsay, 1986). With the exception of their probable antibacterial role, their function in vertebrates is still open to conjecture (Jolles & Jolles, 1984).

Lysozymes are known to cleave the glycosidic bond between the C-1 of *N*-acetylmuramic acid and the C-4 of *N*-acetylglucosamine of bacterial peptidoglycan. It has also been reported to randomly hydolyse β 1-4 *N*-acetylglucosamine linkages (Jolles and Jolles, 1984).

Given the distribution of lysozyme in fish (i.e. in tissues rich in leucocytes and at sites where the risk of invasion is high such as skin, gills and gastrointestinal tract) it is likely that lysozyme provides a protective function in the teleost, especially as it has been demonstrated to be involved in defence against viruses, neoplasms, bacteria, fungi and insects (Dobson, Prager & Wilson, 1984). This defence may not direct but rather mediated via the stimulatory effects on other macrophage functions (Jolles & Jolles, 1984).

Reports on the modulation of the lysozyme activity in fish are rare (Mock & Peters, 1990). However, this study confirms that levamisole can induce increased activities of both mucus and serum lysozyme. No attempt will be made to compare the activity of lysozyme between fish species because there is such a great interspecies variation as shown by Grinde *et al.*, (1988) who reported the lysozyme levels of 12 fish with up to four fold variations between species. Furthermore, variation exists depending on the origin of the sample. Lindsay (1986) assayed lysozyme from the oesophagus, stomach, kidney, spleen, swim bladder and in the mucus and serum. The lowest levels reported were from serum and the highest were from the stomach. This pattern is compatible with a defence function and it seems likely that lysozyme is an integral part of the arsenal of nonspecific defence mechanisms of fish.

As part of the lysozyme assay, the heat labile component of both serum and mucus was studied. It is most likely that the major part of this component was complement since it is inactivated by heat and is found in both the serum and mucus. Complement acts as a membrane attack sequence that may be initiated by either the classical pathway that requires Ig to react with an antigen or via the alternative pathway with stimulation from a variety of substances. Heat-treating mucus and

serum samples prior to conducting the lysozyme assay did reduce the lytic activity but there was not a significant difference between levamisole-treated and control fish, which provides evidence that levamisole had no effect on this heat labile component.

The proliferation responses of different lymphocyte populations to different mitogens have proved to be very useful in distinguishing T and B lymphocytes in mammals (Ellis, 1982). Similar work in fish has demonstrated that T and B lymphocyte populations may also occur in these species. Although there were no significant differences in the stimulation indices demonstrated in this study, as can be seen from Figure 14, there is a general trend in lymphocytes taken from levamisole treated fish which indicates that LPS results in an increased proliferation response. Because LPS is a known B cell stimulant, the results appear anomolous given that levamisole is a T cell stimulant. Levamisole is also known as a prostaglandin blocker and T cells are particularly sensitive to prostaglandin. PHA is a T cell stimulant, thus it would be expected that lymphocytes taken from levamisole treated fish and stimulated with PHA that would show increased proliferation. However, the opposite occurred in my results. It is possible that the PHA cultures are not producing much prostaglandin therefore levamisole has little effect. LPS is a potent monocyte activator and monocytes produce prostaglandins, therefore levamisole could be blocking the prostaglandins produced by the monocytes and acting on the lymphocytes. Thus, these results indicate that in this instance, levamisole appears to be acting as a prostaglandin blocker first and a T cell stimulant second. This pattern of response has subsequently been confirmed in another laboratory (Zelikoff[9], *pers. comm.* 1997).

Finally, the levels of haematocrit and leucocrit serve as a general indicator of fish health. These parameters are often used as confirmation that an immunostimulant is not disturbing the profile of the blood and thus homeostasis of the individual. No significant differences were found between haematocrit or leucocrit levels in treated and control fish. Thus, it may be concluded that the modulatory effect of levamisole does not extend to a modification of the blood profile in this instance.

This study provides strong evidence that *in vivo* treatment with levamisole enhances the nonspecific immune system as measured by phagocytic ability, superoxide anion production, lymphocyte proliferation and lytic lysozyme activity. These findings are in accordance with the previously discussed studies of the effects of levamisole in other fish species. While there is little information available on the implications of

[9] Zelikoff, J. Institute of Environmental Medicine, New York University Medical Centre, New York

these alterations with regard to teleost immune and defence systems and especially defence against extracellular parasites, the mounting evidence for AGD infections suggested that immunostimulation may offer some benefits. As such, the next part of my study examined immunostimulation and the response of Atlantic salmon to infection with *Paramoeba* sp.

The results of this study may prove to be of practical value, beyond that applicable to AGD, given the efficacy of this drug at such low concentrations and the ability to treat large quantities of fish at any given time. Furthermore, the prophylactic use of levamisole may be of value where situations known to result in stress and exposure to disease occur.

CHAPTER 6

IMMUNOMODULATION AND RESISTANCE TO AMOEBIC GILL DISEASE IN ATLANTIC SALMON

Contents

INTRODUCTION

The trials described in the previous chapters confirmed the hypothesis that, under certain circumstances, salmonids that have suffered an attack of AGD are relatively resistant to reinfection. The results of these trials also showed that, as with field outbreaks, some fish do not develop a useful resistance to reinfection with *Paramoeba* sp.. Subsequent rounds of bathing for AGD are often done only to treat this subpopulation of fish; the rest of the fish appear to be relatively resistant except when the challenge is excessive.

There have been a number of unsuccessful attempts to find a chemotherapeutic agent to treat AGD (Alexander; 1991; Cameron, 1994), even though Alexander (1991) and Howard & Carson (1994) have shown that several compounds, including levamisole, inhibit the growth of *Paramoeba* sp. *in vitro*.

It has been previously demonstrated that immunologically impaired animals often respond to levamisole (Symoens & Rosenthal 1977) and there is a large body of evidence, including this study (Chapter 5), supporting the immunostimulatory affect

of this drug in many fish species (Appendix 1). This, together with the *in vitro* amoebicidal and amoebostatic potential of levamisole provided the incentive to trial levamisole as a treatment for AGD.

The series of trials described in this chapter tested the efficacy of levamisole as a treatment for amoebic gill disease in Atlantic salmon.

MATERIALS AND METHODS

Trial One

Initially 140 Atlantic salmon smolts, naïve for AGD, were divided equally and placed in two seawater tanks with 30 AGD-infected (donor) Atlantic salmon post smolts (Figure 16 Procedure A). During the first week, gill lesions characterised by mucoid patches were present on some the naïve fish. By the end of the fourth week severe lesions, coupled with the presence of large numbers of *Paramoeba* sp., were observed on most of the fish. Mortalities began in the middle of the fourth week. After four weeks of infection the remaining fish were removed and divided into the groups listed below:

- 60 fish (30 from each replicate tank) were placed in freshwater for the next four weeks (Figure 16 Procedure B) and were no longer involved in this trial. These fish were being prepared for use in the next AGD challenge trial

- 18 fish (by two replicates) were given an industry-simulated freshwater bath with levamisole at 5ppm (Figure 16 Procedure C)

- 18 fish (by two replicates) were given a freshwater bath without levamisole (Figure 16 Procedure C)

The treated smolts were then returned to their respective tanks, with equal numbers of naïve fish treated in the same manner. That is, two groups of 36 naïve fish given an industry-simulated freshwater bath with or without 5ppm levamisole (Figure 16 Procedure D). No donor fish were added for the second phase of this challenge, that is, any recrudescence of AGD would result from residual infection in the treated fish. To ensure this, tanks were completely drained, scrubbed and washed and then filled with freshwater for two hours. This would kill any *Paramoeba* present in the experimental tank system (Howard *et al.*, 1993)

Weekly for four weeks the severity of infection was monitored.

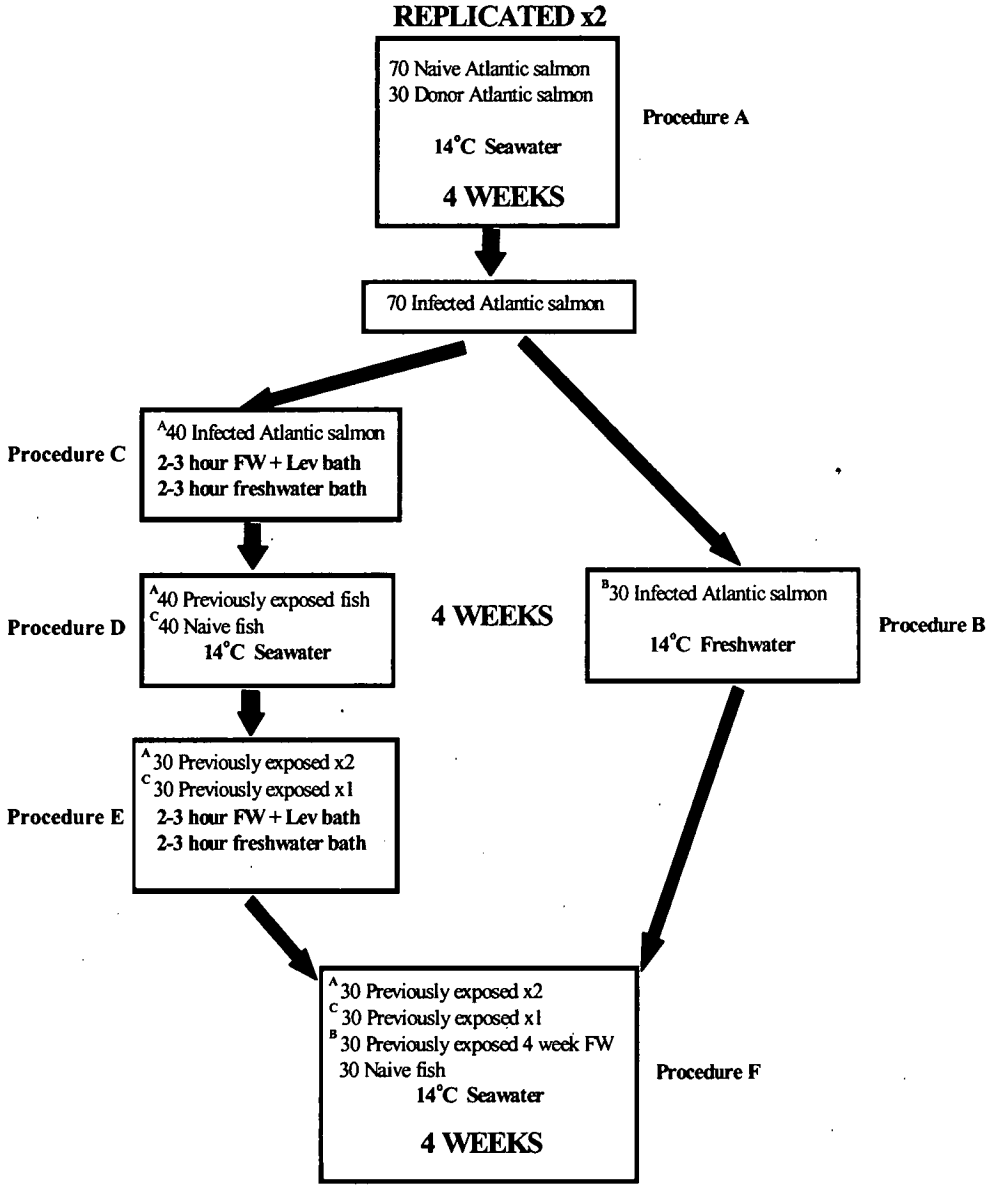
At the conclusion of this trial all surviving fish were given another industry-simulated bath with or without 5ppm levamisole (same as the previous treatment for each group) and these became the 'previously exposed x1' and previously-exposed x2' groups for the next challenge (Figure 16 Procedure E).

Trial Two

The next AGD challenge trial involved eight different groups of fish that had been infected for varying periods of time and treated for AGD in different ways. For each of the following groups, half were treated with a freshwater bath only and the other half with a freshwater-levamisole bath (concentration 5ppm): 60 naïve fish, 60 fish that had been exposed for four weeks and given a two hour bath, 60 fish that had been exposed twice and given a two hour bath at the end of each four week period, and 60 fish which had been exposed once and then kept in freshwater for four weeks (Procedure F). Each of the eight groups was equally divided and placed into their respective tanks. No donor fish were added for this challenge. See Figure 16 for flowchart of experimental method and Table 3 for detail of numbers of fish per treatment group.

Weekly for four weeks the severity of infection was monitored.

Figure 16 Experimental procedure used in AGD challenge trials



Note: matching superscripts denote the same group of fish as they move through the experiments

Table 3 Summary of experimental groups and results for all levamisole AGD trials

Trial no. / Treatment	Levamisole Dose mg/L	Experimental Procedure	Sample size	Lesion No. at week 4	Mortalities	RPP%
1/PE Lev	5.0	A-C-D	36	8.4	1	97.2
1/PE FW	0	A-C-D	36	17.9	1	97.2
1/Naïve Lev	5.0	D	36	16.1	2	94.4
1/Naïve FW	0	D	36	24.1	9	77.8
2/PEx4 Lev	5.0	A-B-F	30	5.2	0	100
2/PEx 4 FW	0	A-B-F	30	4.0	0	100
2/PEx2 Lev	5.0	A-C-D-E-F	30	3.2	0	100
2/PEx2 FW	0	A-C-D-E-F	30	4.9	0	100
2/PE Lev	5.0	D-E-F	30	7.0	2	93.3
2/PE FW	0	D-E-F	30	N/A	30	0
2/Naïve Lev	5.0	F	30	21.5	7	76.6
2/Naïve FW	0	F	30	N/A	30	0
3/PE Lev	5.0	A-C-D	36	4.0	0	100
3/PE Lev	2.5	A-C-D	31	5.4	0	100
3/PE Lev	1.25	A-C-D	33	6.1	0	100
3/PE FW	0	A-C-D	34	25.9	6	82.4
3/Naive	0	D	40	39.8	15	62.5

PE= previously exposed, FW= freshwater bath only, Lev= freshwater bath + levamisole, N/A=too few fish remaining for lesion numbers to be valid. See Figure 16 for experimental procedures A, B, C, D, E & F. RPP- relative percentage protection (or the percentage of fish surviving the trial).

Trial Three

Given that it is desirable to minimise the chemicals being used in the production of animals for human consumption, this challenge was designed to test the efficacy of two concentrations of levamisole lower than that used in the previous trials.

Initially, two replicates of 80 Atlantic salmon smolts (total of 160 fish) were divided equally and placed in two seawater tanks together with 40 AGD-infected post-smolts. After four weeks these fish were further divided into four groups (20 fish/group/tank). One group acted as a control with the other three groups being treated with one of the following concentrations of levamisole: 5ppm, 2.5ppm or 1.25ppm. Another group of 40 naïve fish were given only a freshwater bath and divided equally between the two replicate tanks. No donor fish were added for the second part of this challenge.

Each week for four weeks post-treatment the severity of infection was monitored.

There were unequal numbers of fish in each group of this trial because the covers were removed from the experimental tanks just after the levamisole and freshwater treatments were done. A number of fish jumped from the tanks and could not be replaced without a six week delay. Given that there were still enough fish to conduct a statistically robust trial it was decided to continue with uneven numbers of fish.

RESULTS

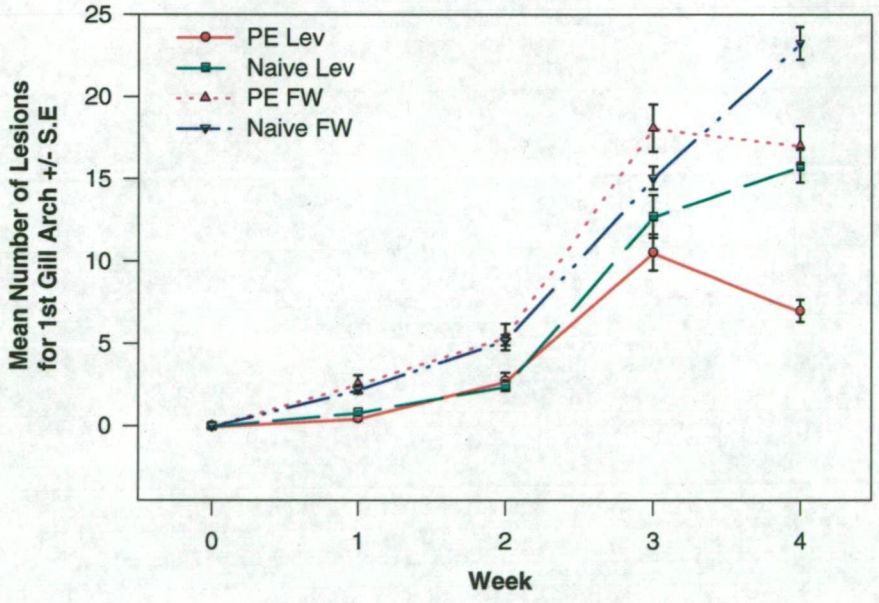
Trial One

It became apparent in this trial that fish which were experiencing their second wave of infection after being given an industry-simulated freshwater bath did not exhibit the same resistance to reinfection as fish that were allowed to recover for four weeks in freshwater before being re-exposed. However, when fish experiencing their second wave of infection were bathed in freshwater and levamisole, demonstrable resistance was significantly increased (F_{calc}=51.73, d.f=1, $p<0.0001$). This was particularly apparent at three and four weeks post-exposure to *Paramoeba* sp. Lesions on levamisole-treated salmon experiencing their first wave of infection were considerably reduced compared to the number of lesions on their freshwater bathed counterparts. In fact, those fish that had been treated with levamisole prior to experiencing their first wave of infection were more resistant to infection than fish that had been previously exposed and treated only with freshwater, as indicated by the number of lesions on the gills. Tukey's groupings and LSD differentiated these

two groups of fish. Mortalities due to AGD were significantly reduced in levamisole treated fish experiencing their first wave of infection compared to their freshwater bathed counterparts, but there was no difference in the number of mortalities in fish that had been previously-exposed whether or not they had been treated with levamisole.

The changes in lesion numbers over the four-week challenge period are shown in Figure 17 and a summary of mortality, relative percentage protection (RPP) and lesion data for all challenges is presented in Table 3. Experimental procedures may be reviewed in Figure 16.

Figure 17 AGD lesion patterns in fish treated with a freshwater and levamisole bath or a freshwater bath only



PE = Fish that have been previously exposed; Naïve = Fish that were naïve in relation to AGD prior to the trial; Lev = Fish were treated with a freshwater and levamisole bath; FW = Fish were treated with a freshwater bath without levamisole

Trial Two

It was noted that the continual passage of AGD through susceptible fish apparently raised the virulence of *Paramoeba* sp. By the end of this trial, the effects of this

increased virulence were evident. While the pattern of infection remained similar to that of previous trials, the magnitude of infection was greater, thus affecting the outcome compared to the previous trial. Indeed, because of the mortalities that occurred in naïve fish and fish that had been exposed to AGD on one occasion, but treated with freshwater only, these two groups were withdrawn from the trial before four weeks had elapsed. However, their levamisole treated counterparts displayed a RPP of 76.6% (naives) and 93.3% (previously exposed) (Table 3).

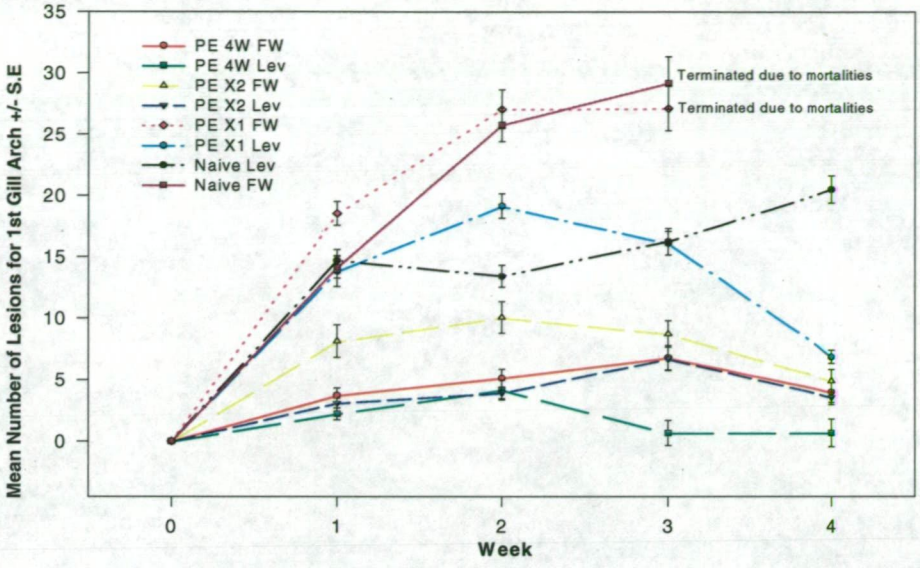
As demonstrated in the previous trial, levamisole treated fish were significantly more resistant to reinfection than fish treated only with freshwater (F_{calc}=191.04, d.f=1, $p<0.0001$; Figure 18). Tukey's groupings and LSD differentiated between fish experiencing their first and second waves of infection that had been treated with levamisole when compared to their freshwater treated counterparts. Furthermore, fish that had been treated with levamisole and exposed to AGD for the first time performed significantly better than fish that had been previously exposed to *Paramoeba* sp. and treated only with freshwater, as differentiated by Tukey's grouping and LSD.

Levamisole treatment did not significantly affect the outcome (ie the condition of the fish by the end of the trial) for fish that had experienced two waves of infection, but it did significantly reduce the number of lesions one and two weeks post exposure, as defined by Tukey's grouping and LSD.

Levamisole treatment did not affect the outcome or lesion numbers for fish that had been returned to freshwater for four weeks after initial infection; Tukey's groupings and LSD did not differentiate between these groups.

The differences in lesion numbers between the eight groups may be seen in Figure 18. A summary of all other data can be reviewed in Table 3.

Figure 18 AGD lesion patterns in fish with varying exposures to AGD and treated with a freshwater and levamisole bath or a freshwater bath only

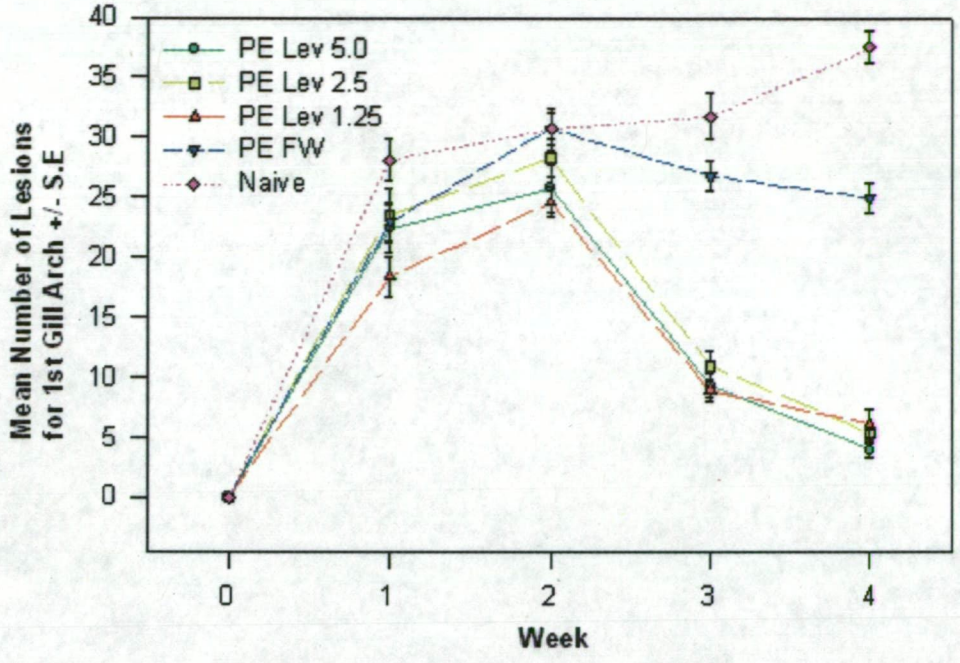


PE 4W = Fish that had been previously exposed to AGD and kept in freshwater for four weeks after, PE X2 = Fish that had been exposed to AGD twice previous to this trial, PE X1 = Fish that had been exposed to AGD once previous to this trial, Lev = Fish that were treated with a freshwater and levamisole bath; FW = Fish that were treated with a freshwater bath only

Trial Three

In trial three, groups of fish were treated with different concentrations of levamisole (Figure 19). There were no significant differences between lesion numbers or RPP for any groups treated with levamisole (Tukey's groupings did not distinguish between any of the three levamisole treatments). However, the groups given only a freshwater bath had more lesions and greater mortality than those treated with levamisole (Table 3). As demonstrated in previous challenges, the naïve fish and previously exposed fish bathed in freshwater only displayed significantly increased lesion numbers and mortalities when compared to previously exposed fish given a freshwater plus levamisole bath (Tukey's differentiated between the three levamisole-treated groups and the naïve group).

Figure 19 AGD lesion patterns in fish treated with varying concentrations of levamisole



PE = Fish that had been infected with AGD prior to this trial, Naïve = Fish that were naïve in relation to AGD prior to this trial, Lev 5.0 = Fish were treated with a freshwater and 5ppm levamisole bath, Lev 2.5 = Fish were treated with a freshwater and 2.5ppm levamisole bath, Lev 1.25 = Fish were treated with a freshwater and 1.25ppm levamisole bath, FW = Fish were treated with a freshwater bath only.

DISCUSSION

It is now well established that fish that have been previously exposed to AGD show varying degrees of resistance to reinfection, dependent on treatments used. In the pilot trial presented in Chapter 4, it was shown that fish that had been exposed to AGD, kept in freshwater for four weeks and then re-challenged displayed a high level of resistance to reinfection. It is interesting that this same level of resistance is not reached when fish are given a single, industry-simulated freshwater bath of two to three hours. Even the small decrease in lesion numbers often seen in the fourth week after the freshwater bath is illusory due to the coalescence of lesions when fish exhibit such high numbers, as well as the death of the most severely affected fish which carry the highest number of lesions. Thus, it appears that the fish which are exposed to *Paramoeba* sp. for the first time and then bathed for two to three hours do not develop appreciable signs of resistance to reinfection. In fact, their ability to

cope with *Paramoeba* sp. challenge seems little different from that of fish that are experiencing their first wave of infection.

In contrast, fish which have been given two industry-simulated baths display a high level of resistance to reinfection. It is notable that there was no significant difference in the outcome between this group of fish and those that had remained in freshwater for four weeks. The small difference that is seen in the early weeks of the challenge may be explained by the fact that fish which are given a two to three hour bath probably still maintain low levels of amoebae on their gills after a bath, while fish which have been held in freshwater for four weeks are completely cured before being re-challenged.

While there were significant decreases in mortalities and lesion numbers in most of the groups of the fish treated with levamisole, it was those fish experiencing their second wave of infection (i.e. fish which had been previously infected and given a freshwater bath before being re-exposed) benefited most from the treatments. This is possibly the result of combined responses to both the infection and the immunomodulator. Siwicki *et al.* (1992) have previously demonstrated that where antigens are themselves nonspecific defence stimulators, a synergistic effect was evident when levamisole treatment was also used. It would therefore appear that fish that had experienced even more waves of infection presumably develop a high level of nonspecific defence as a result and the synergistic effect of levamisole is not so apparent.

It is notable that fish which have been infected and allowed to recover fully in freshwater for four weeks display significant resistance to reinfection whether or not they are treated with levamisole. It appears that stimulation of the nonspecific defence system following one wave of infection coupled with the fact that the fished gills would have recovered completely from the infection ensures that these fish are almost completely refractory to reinfection. This indicates that re-establishment and development of AGD is the result of interplay between immune and defence responses (especially the nonspecific defense system, gill health, number and virulence of *Paramoeba* sp. and the environmental conditions (especially temperature and salinity)). In this context, it is also relevant that levamisole has been reported to significantly enhance the healing process in mammals (Symoens & Rosenthal, 1977). This enhanced healing was attributed to the increase of neutrophil and macrophage migration to the damaged area, and it is reasonable to assume that a similar mechanism may exist in teleosts.

To put the various responses to levamisole in context it is suggested that the factors involved are as follows:

- Fish infected for the first time and given a two hour freshwater bath only will develop a moderate increase in their nonspecific defense system and will have mucus and amoeba removed from their gills. However, the lesions of gill hyperplasia and inflammation will remain to attract amoebae (Nowak & Munday, 1994; Zilberg[10] *pers. comm.*, 2000) and some amoebae may survive within cystic lesions present in the gills (Munday *et al.*, 1990)

- Fish infected for the first time and given a two hour freshwater bath containing levamisole will experience a very much enhanced nonspecific defence response, together with removal of mucus and amoebae, and, therefore, there will more likely be resolution, rather than persistence of infection and resultant lesions.

- In fish that have been previously exposed on two occasions and given two industry-simulated baths there are lesions still present but the nonspecific immune response has been augmented to a sufficient level to allow recovery. In this instance levamisole provides only a temporary advantage of a slightly higher resistance to reinfection in the early weeks; probably only indicative of the fewer lesions on the gills because of the previous levamisole treatment.

- Fish that have been infected for the first time and allowed to recover in freshwater for four weeks have gills that are in excellent condition when re-exposed, so while their defence response may not remain at as higher levels as the fish that are re-exposed immediately after treatment, the condition of the gills compensates for this.

A central factor in all of the above observations was that in challenges where fish had been given an industry-simulated freshwater bath, no 'donor' fish were required to re-establish infection, thus indicating some carry-over by treated fish. This is in contrast to the trials in which fish are held in freshwater for four weeks. This evidence indicates that there may not necessarily be a reservoir of infection in the immediate environment, but rather there are enough viable stages left on the fish after a two to three hour bath that reinfection is immediately facilitated once the fish are returned to sea water.

Throughout these trials it became apparent that the group of fish that did not demonstrate resistance to reinfection remained, albeit at a reduced level. On administration of levamisole to these groups the number of 'non-responders' was

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reduced but never eliminated. Given that there is a high individual variation in defence responses between fish it is likely that levamisole can, in some instances, restore a sufficient defence mechanism for a demonstrated resistance to *Paramoeba* sp. but it does not appear to be able to totally restore normal function in all immunological sub-performers. It is believed that this too, may be influenced by the interplay of factors as described above, but in particular, by the severity of infection and condition of the gills at the time of treatment. In regard to this group of fish it would be of particular interest to determine the absence or presence of that 'factor' discussed below, which has been reported in humans, rabbits and mice treated with levamisole (Symoens & Rosenthal, 1977). A similar scenario would appear to apply equally well to AGD infections in fish.

Even though levamisole has been shown to increase antibody titre in fish (Jeney & Anderson, 1993; Siwicki, 1987), as previously discussed in Chapter 5, there is no apparent relationship between detectable antibodies against *Paramoeba* sp. and resistance to AGD (Findlay, Helders, Munday & Gurney, 1995; Akhlagi, Munday, Rough & Whittington, 1996), at this stage, this potential aspect of the drug's immunomodulatory repertoire is not thought to be relevant. On the evidence to date, it appears that the nonspecific defence system is the most important for Atlantic salmon exposed to AGD.

With regard to the large scale use of levamisole, results from farms have been variable. In the first summer that levamisole was used, no AGD occurred as a result of a prolonged period of low salinity water covering the farms. The second trial gave variable results. In some instances there appeared to be significant protection provided to fish treated with levamisole. In a pen of fish treated with levamisole, 30% displayed AGD lesions and no mortalities were recorded, while in the untreated pen, 90% of the fish displayed lesions and mortalities occurred. However, in other instances, there did not appear to be any effect.

There are a number of reasons why levamisole has variable effects when used in the farm situation. These too are thought to be dependent on the complex interplay of factors discussed above.

- In the farm situation, bathing is undertaken sometimes prophylactically and at other times when only a few lesions can be seen in some fish. If the nonspecific defence system is stimulated by the presence of *Paramoeba,* as has been shown in Chapter 4, and the fish are treated before being exposed to a sufficient dose, then this stimulation would not be apparent. More than this, the synergistic effect that may be seen when levamisole is used in conjunction with an immunostimulating antigen would not occur.

- Levamisole may only be of benefit where the attacks of AGD are moderate to severe. The reasons for this have been discussed previously.

- Because of practical constraints arising with the transfer of tens of thousands of fish from one cage to another, some fish may be left in the freshwater bath for up to seven to eight hours. This has been demonstrated to be potentially detrimental to the health of the gill (Munday[11], *pers. comm.* 1997) and may even provide areas which are preferential attachment sites for amoebae.

- The dosage used and the administration of it may not have been done correctly in some circumstances. As discussed in Chapter 5, levamisole is immunosuppressive when used in high doses and ineffective at low doses. Furthermore, it is inactivated at alkaline pH.

The results from the predominantly experimental studies described in this thesis have still to be confirmed under fish farm conditions using statistically valid methods.

The next part of this discussion will look at the mode of action and clinical effects of levamisole in an effort to define some of the reasons why this substance provides protection against *Paramoeba* sp. in Atlantic salmon. Levamisole has been shown to be effective in treating a number of diseases and dysfunctions and some of these may be relevant in hypothesising the mechanism of action in relation to *Paramoeba* sp.

In particular, levamisole is known to treat chronic and recurrent infections, primary and secondary immune deficiency states, allergic disorders, rheumatic diseases, gastrointestinal diseases and skin disease (Symoens & Rosenthal, 1977). It is also very active in a variety of inflammatory conditions (Pillar, 1975). Given that the response to AGD is primary inflammation and hyperplasia, the effect of levamisole may extend beyond modulation of the defence system to a more specific affect on the inflammatory response.

During levamisole treatment, many studies have shown that immunologic functions improved and in most cases T cell and phagocytic functions were heightened (Symoens & Rosenthal, 1977). As a rule however, there was no good correlation between immunologic and defence system functions and therapeutic effects. None of the variables of cellular immunity represents a reliable means of predicting

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therapeutic responses to levamisole treatment (Desidero & Rankin, 1986). This makes it's effectiveness as a treatment for AGD even more intriguing.

In particular, Symoens & Rosenthal (1997) presented the results of research which demonstrated that serum from levamisole treated humans, mice and rabbits contained at least one dialyzable factor that could be isolated and lyophilised. The factor was only found in animals which responded to levamisole and was not observed in non-responders or untreated animals. The factors when injected in untreated animals mimicked the effect of levamisole including enhanced carbon particle clearance and converted non-responders into responders. The factor was not a complement factor nor a levamisole metabolite. It was not found in animals treated with other stimulants such as LPS. Investigation into the occurrence of this 'factor' in Atlantic salmon may provide some answers relating to the group of non-responders. As mentioned above, research into what this 'factor' is and whether it occurs in Atlantic salmon would provide many answers on the AGD pathogen-host relationship.

The increased protective effect provided by levamisole, that is seen in Atlantic salmon, cannot be explained by a direct effect on invading organisms; Howard & Carson (1993) demonstrated that it takes at least 10ppm levamisole to kill *Paramoeba* sp. *in vitro* but it may, *inter alia*, be related to a modulation of lesion and wound healing. As previously stated, evidence in the literature, together with results presented Nowak & Munday (1994) and Zilberg (*pers. comm.*, 2000) suggest that gill hyperplasia and epithelial disruption may attract amoeba. Therefore, any treatment that reduces these would presumably result in a less attractive environment for the amoeba. Furthermore, if as has been suggested by the numerous workers in the field including Munday *et al.* (1990), that the mucus production facilitates removal of the amoebae then there is evidence that levamisole also contributes positively in this area (Zilberg, *pers. comm.* 2000).

Indeed, there is evidence that levamisole may enhance the nonspecific defence system, the specific immune system and the overall health of the gill. From the evidence presented in this thesis, levamisole can make a vital contribution to the resistance demonstrated by Atlantic salmon exposed to *Paramoeba* sp.

CHAPTER 7

GENERAL DISCUSSION

At the commencement of this project, there were still significant areas of knowledge in relation to the disease and causative agent that were anecdotal and had not been tested by rigorous scientific inquiry. *Paramoeba* sp. had been isolated and could be cultured, but disease could not be reproduced using these cultured organisms. Fish had been experimentally infected using a cohabitation technique but these could not be maintained for longer than 10-14 days. The pathology of the disease had been well reported, and there was some work being done to develop diagnostic tests for use in both the farm and laboratory environment, but only limited immunological studies had been undertaken.

It is interesting to note that, to date, the *Paramoeba* sp. causing AGD has not gained as much attention overseas as it has in Australia. This is probably due to the fact that, until recently, AGD had limited effects in fish stocks overseas. Much of the literature from overseas (as discussed in Chapter 1) is limited to descriptions of AGD, the species that it affects and in some cases it extends to some histological work. It is likely that the amount of research being conducted overseas will increase and the type of research will change from reporting occurrence and general pathological effects to experimental work on epidemiology and treatment of AGD as the effects of the disease increase overseas.

As AGD became more limiting to the growth of the Tasmanian salmonid industry, research on this disease began assuming greater importance. Any strategy that would eliminate or reduce the number of freshwater baths for the control of AGD would be of significant commercial value. It was the anecdotal observation made by industry, that fish that had been previously infected with AGD appeared to demonstrate some resistance to reinfection that precipitated this project. The scientific demonstration of resistance to reinfection would pave the way for informed research to be conducted on methods of engendering protection against AGD in susceptible fish species.

The most important findings emanating from this project were the demonstration of the resistance to infection with AGD in fish that had been variously exposed to the disease and the fact that this could be modulated. These developments facilitated the conduct of trials that further clarified and enhanced this pivotal development in the

understanding of AGD. Also, this is the first record of the use and efficacy of levamisole as an immunodulator of the nonspecific defence system in Atlantic salmon.

To put things in context, the following section of discussion will present my hypotheses on the events leading to the infestation of *Paramoeba* on the gills of Atlantic salmon, how infestation occurs and what factors are involved in resistance.

Like all pathogenic organisms, it is most likely that *Paramoeba* sp. virulence is related to a number of amoebic components and host factors. It is a well reported observation that the amoebic molecule chiefly responsible for adherence to target cells is a lectin. As shown by Zilberg, Gross & Munday (2000) *Paramoeba* sp. can be found in association with normal gill epithelium. This is more than likely mediated via a lectin, probably a galactosamine and/or glucosamine specific lectin. Once initial attachment has been made it seems likely that just as for *Entamoeba histolytica*, localised actin polymerisation would correlate with the extension of pseudopodia.

Pseudopodia of the *Paramoeba* sp. have been demonstrated in the intercellular junction regions between adjacent cells of gill epithelium (Munday *et al.*, 1990) and where this happens, irritation would ensue. Inflammation and epithelial hyperplasia results, whether it be from the mechanical damage inflicted by the pseudopodia or extracellular products of the amoeba is not known. The hyperplastic tissue that is seen in affected gills is particularly reactive, with a high cellular turnover, presumably trying to rid the area of the irritant. This results in an increasing area of immature surfaces and most likely a preferential attachment site for the *Paramoeba*, either because there are physically more areas to occupy or because the sugar moieties of immature cells preferentially attract amoeba. While it is recognised that *Paramoeba* may occur on normal gill tissue, it is well accepted by workers in the field (eg Roubal *et al.*, 1989, Nowak & Munday, 1994) that *Paramoeba* preferentially attach to hyperplastic areas of the gill.

All of these effects lead to increased bacterial loads, as seen by Garland (*pers. comm.*, as cited in Munday *et al.*, 1993), and more food for the amoeba. This establishes the cyclical escalation of disease, so often seen in parasitic infections of this nature. Until 'something' breaks the cycle, disease progresses until the affected fish dies. Spontaneous resolution of AGD in the absence of treatment has not been observed in this, or any other study.

Beginning with the anecdotal reports of the development of resistance in fish previously exposed to disease, through to the completion of my project and beyond, an issue that has exercised my mind is how a specific humoral response would actually occur. Other than a single report of pseudopodia being observed between the cellular junctions of hyperplastic tissue, there is little evidence to suggest that the bodily barriers are actually breached by whole *Paramoeba*. Therefore some other mechanism must be involved if antigens are to be presented to antigen processing cells.

There are three scenarios that may lead to the development of a humoral immune response:

- Antigen presenting/processing cells may come into contact with pseudopodia that are inserted through cellular junctions. This may be a more regular event than reported, as fixation and histology techniques may result in disruption of the attachment (often areas on the gill epithelium that exactly match the shape of an amoeba close by can be found).

- The amoebae that may be seen in the cysts that form in hyperplastic tissue (Plate 3; Plate 4) presumably would die, thus making antigen available to invading macrophages.

- Alternatively, soluble amoebic antigens may leak into the circulation via the disrupted epithelium. The events that follow initial antigen recognition have been reported in Chapter 1.

Once antibodies have been produced, there are no guarantees that these will be protective and evidence from this study together with others mentioned in previous chapters certainly alludes to this. If protective antibodies are formed, are these of local or systemic origin?

If the specific immune system is not providing the protection against reinfection with *Paramoeba* that has been reported in this study, what are the other options?

It is a characteristic of an immune response in a vertebrate that the response time to a second exposure is less than to the first; this is known as anamnesis. However, the non-immunoglobulin humoral response factors of vertebrates seem to lack

anamnestic properties. But as with all statements in biology, there are exceptions to the rule. One possible reason for the retention of many nonspecific humoral defence mechanisms by fish is the reduced (primitive) immunoglobulin responses compared to higher vertebrates (Alexander & Ingram, 1992).

On infection with *Paramoeba*, the nonspecific immune system is heightened as indicated by the phagocytic parameters measured in this study. Whether this is the result of stimulation via amoebic surface coat components, or via components of bacterial cell walls, is unknown at this stage. While the stimulation remains, the nonspecific defence system remains active, this presumably holds true for some period after the stimulation is removed also. If the stimulation is again provided before the defence system has returned to basal levels, this may result in a stepwise increase and ultimately a reaction sufficient to display a demonstrable resistance to reinfection.

In all cases in this study, the immunostimulant levamisole heightened the nonspecific defence response, and although not measured, as discussed in Chapter 1, it is likely that it also stimulated the specific immune response. However, because salmonids are primitive fish compared to many of the other teleosts it is conceivable that their dependence on the nonspecific defense mechanisms is far greater than for the higher teleost. From what we know in the literature and what this study demonstrated, levamisole may act in the following ways:

- Given the apparent importance of lectins in both adherence and pathogenesis of amoeba, if these can be inactivated then adherence ability and pathogenesis would logically be decreased. As lysozyme, the lytic agent that cleaves various sugar moieties increases in levamisole treated fish, there is evidence to suggest that adherence via a galactosamine/glucosamine lectin may be inhibited. Either via an increase in the availability of preferred ligands due to the lysis of bacterial cell walls (ie gill epithelium is 'flooded' with exogenous lectin substrate) or because links established between the amoeba and the gill are split.

- Reactive oxygen and nitrogen intermediates (ROI & RNI, respectively) have been reported to participate in the destruction of, inter alia, bacteria and protozoa. Once again levamisole treated fish displayed significantly heightened levels of ROIs as well as a capacity to sustain this for increased periods. The most likely scenario in relation to the efficacy of these products is that the pseudopodia inserted between gill cells are 'burnt off' after contact with these cytotoxic

factors and would be removed from the gills. The enhanced state of healing and wound repair engendered by levamisole may act to counteract the toxic cellular effects of these ROIs and RNIs.

- Alternatively, and most simply amoeba may be removed from the gills via the production of mucus. As previously discussed, levamisole treatment appeared to increase mucus production thereby acting in addition to the benefits of the freshwater bath (which probably acts mainly to destabilise the seawater stable gill mucus, thus not only is the fish induced to have a 'big sneeze' it is stimulated to rapidly replace the lost mucus with fresh mucus rich in lysozyme).

Any of the above scenarios would 'break' the AGD cycle.

At the culmination of this project a number of observations stood out as being prospective candidates in the continuing efforts to develop a more efficient and less costly treatment and/or cure for AGD.

I believe that amoebic lectins are the key to the pathogenesis of *Paramoeba* sp. Valuable developments can be made by looking at the different sugar properties of cultured and wild *Paramoeba* and also those associated with the gill tissue, both normal and hyperplastic.

Work is required to establish whether extracellular products play a part in the development of resistance and to what extent. At this point in time, no assays have been conducted that would detect antibodies developed against extracellular products.

Little is known of how *Paramoeba* interact with the gill. If the mode of interaction can be established work can be focussed on plausible scenarios that follow.

Given the success of levamisole as a treatment for AGD in the experimental situation it would be prudent to look at different immunomodulators under similar conditions. By a process of elimination and establishing the modes of action of each modulator, a better understanding of the defence and/or immune mechanisms involved in AGD infections can be gained.

Still, it must be recognised that the knowledge of the immunological relationships between fish and their parasites is small in comparison with that concerning bacteria and viruses. Woo (1992) summarised the most significant of these:

- Problems encountered in laboratory manipulation of parasites in comparison to bacteria and viruses
- The difficulties of *in vitro* and *in vivo* culture of organisms
- Intrinsic complexities of the parasites and their often complex lifecycles.

The project presented in this thesis has contributed knowledge to each of these facets in relation to AGD. The success of defining resistance to AGD reinfection in Atlantic salmon and also in overcoming the hurdle that prevented large scale, long term AGD trials being conducted, by establishing a protocol for the *in vivo* continuous maintenance of *Paramoeba* sp, will contribute to much of the subsequent work that will be carried out. Principally it has provided a reliable source of parasites for conducting AGD trials at will, as well as for developing diagnostic techniques. It has established important immunological assays and experimental designs for research into defence and immune modulation. It will also facilitate the evaluation of the host-parasite relationship, which is now fundamental to the continuation of AGD research.

A comment of the applicability of this research to the field situation is warranted.

Because the expression of disease involves many interactive processes between host, pathogen and environment, it is important to remember that any treatment regime needs to be optimised for the current situation. None of this data should be misconstrued to indicate that levamisole is the answer to the amoebic gill disease problems in Tasmania. It does however indicate that the natural response of certain groups of salmon can be augmented under the correct administration and conditions in order to increase resistance and survival against amoebic gill disease. Most importantly it provides the sound scientific basis to continue AGD research in a well-considered and calculated manner.

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APPENDIX I

SUMMARY OF IMMUNOSTIMULATION STUDIES IN FISH

(Turn over for Table 1)

Table 1 Summary of immunostimulation studies in fish

Immunostimulant	Administration	Dose	Fish species	Results	Authors
Abalone extract	Injection	10mg/fish	Rainbow trout	↑ phagocytosis	Klontz & Anderson, 1970
	Injection		Rainbow trout	↑ disease resistance	Sakai et al., 1991
Achromobacter stenohalis cells	Injection		Char	CL ↑, Complement ↑	Kawahara et al., 1994
Alginate	Injection	10-40 mg/kg via Artemia	Carp	↑ in survival when challenged	Fujiki et al., 1994
	Oral		Turbot	↑ in survival when challenged	Skjermo et al., 1995
	In vitro		Carp	↑ head kidney phagocyte migration and activity	Fujiki & Yano, 1997
	Oral		Atlantic salmon	↑ lysozyme	Gabrielsen & Austreng, 1998
Aluminium hydroxide	Injection	0.1 ml	Atlantic salmon	↑ survival when challenged	Lillehaug et al., 1992

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Oral and anal	(1:1 w/w ratio with 10 $mg.ml^{-1}$ HGG)	Tilapia	↑ level of antigen absorptioninto plasma. No effect on antibody titre	Jenkins et al., 1994
Aluminium phosphate	Injection	0.1 & 0.2ml	Atlantic salmon	↑ antibody titre	Midtlyng et al., 1996
	Injection	0.1 ml	Atlantic salmon	↑ antibody titre & survival when challenged	Erdal & Reitain, 1992
	Injection	0.1 ml	Atlantic salmon	↑ survival when challenged	Lillehaug et al., 1992
	Oral/ Injection		Salmonids	↑ antibody and resistance	Klontz & Anderson, 1970
	Immersion & Injection	$5g/kg^{-1}$ feed	Tilapia	↑ antibody titre, mitogenic response, macrophage migration inhibition & survival when challenged	Ramadan et al., 1994
Ascogen	Injection	0.01 ml	Rainbow trout	No effect on antibody titre	Grayson et al., 1987
Bacille Calmette Guerin	Immersion	1/300 dilution	Rainbow trout	↑ bacterial clearance	Grayson et al., 1987

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Oral	0.2%	Channel catfish	↑ macrophage & neutrophil migration & phagocytic activity. No effect on survival when challenged or antibody titres	Duncan & Klesius, 1996a
B-glucan	Injection	0.1 ml	Turbot	↑ respiratory burst & leucocyte killing ability. No adjuvant effect on bactericidal or lysozyme activity	Santarem et al., 1997
	Injection	2-10mg.kg^{-1}	Common carp	↑ survival when challenged, phagocytic activity & activation of alternative complement pathway	Yano et al., 1989
B-1,3-glucans	Injection	50 & 70 ug	Channel catfish	↑ phagocytic 7 bactericidal ability, ↑ survival when diseased	Chen & Ainsworth, 1992
	Injection	2-10mg.kg^{-1}	Yellowtail	↑ survival when challenged, serum complement, lysozyme & phagocytic activity	Matsuyama et al., 1992
	Oral	1 g.kg^{-1} diet	African catfish	↑ respiratory burst & bactericidal ability	Yoshida et al., 1995
	Injection	1.0 & 0.7ml	Atlantic salmon	↑ lysozyme and complement activity	Engstad et al., 1992

Immunostimulant	Administration	Dose	Fish species	Results	Authors
B-1,3 & B-1,6-glucans	Injection	1 ml	Rainbow trout	↑ O^{2-} and bactericidal activity. No effect on lysozyme activity	Jorgensen et al., 1993b
	Oral	0.2 g/100 g feed	Rainbow trout	↑ O^{2-} and phagocytic and myeloperoxidase activity, immunoglobuline level and survival when challenged	Siwicki et al., 1994
	In vitro		Atlantic salmon	↑ Phagocytic and acid phosphatase activity	Dalmo & Seljelid, 1995
	Injection	5 $mg.ml^{-1}$	Atlantic salmon	↑ antibody level. No effect on survival when challenged.	Aakre et al., 1994
B-1,3-M-glucans	Oral		Atlantic salmon	No adjuvant effect on antibody titre.	Midtlyng et al., 1996b
B-1,3-M-glucans (Macrogard®)	Injection and oral	250 ug & 0.1% or 1%	Channel catfish	↑ antibody titres & O^{2-} activity. No effect on lysozyme & survival when challenged	Ainsworth et al., 1994
B-glucan	Bath/oral	100mu g/ml & 0.1-0.5%/food kg	Sturgeon	↑ nonspecific defence, ↑ phagocytosis, ↑ ROI	Jeney et al., 1994a
	Bath	1.0-5.0mg/kg	Sea bass	↑nonspecific defence, ↑phagocytosis, ↑ROI	Jeney et al., 1994b

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Oral	6 &60ug/kg BW	Rainbow trout	↑ disease resistance	Matsuo & Miyazono, 1993
Bifidobacterium thermophilum	Bath	10-12 ug/ml	Cunners and strlped bass	↑ humoral antibody	Robohm & Ra, 1986
Cadmium (Cd)	In vitro	1 ug/ml	Rainbow trout	↑ chemiluminescent response.	Elsasser et al., 1986
	Oral	2.7g/100g feed	Rainbow trout	↑ O^{2-} activity, myeloperoxidase level, neutrophil killing activity & survival when challenged.	Siwicki et al., 1994
Candida utilis	Oral		Trout	Phagocytosis ↑, ROI ↑	Sakai et al., 1995a,b
C. butyricum	Injection	100 $mg.kg^{-1}$	Rainbow Trout	↑ phagocytic activity, CL response & survival when challenged	Sakai et al., 1990
Chitin	Injection		Trout	Phagocytosis ↑, Lysozyme →	Sakai et al, 1992
	Oral		Trout	ROI ↑, Phagocytosis ↑	Siwicki et al., 1994
Chitosan	Injection		Trout	ROI ↑, Killing ↑	Anderson et al., 1995
	Injection	0.5 ml	Rainbow trout	Opportunistic infection.	Munn & Trust, 1983

Immunostimulant	Administration	Dose	Fish species	Results	Authors
Complete Freunds Adjuvant	Injection	0.25 mg/kg	European eel	Lymphophilia.	Van der Heijden et al., 1993
Con A	Injection	0.1 ml/fish	Coho salmon	↑ resistance to disease	Olivier et al., 1985
CFA Modified	Injection	5 mg (2 days after antigen)	Eel	↑ phagocytosis and antibody	Sigel et al., 1983
Ecteinscidia turbinata extract	Oral	100-1000 mg/kg	Rainbow trout	↑ CL and phagocytic activities and survival when challenged	Yoshida et al., 1993
EF203	Injection	0.1 & 0.05 ml	Rainbow trout	↑ phagocytic activity & ROI response. No effect on agglutinating titre	Sakai et al., 1995
	Oral		Trout	Phagocytosis ↑, CL ↑	Yoshida et al., 1993
	Oral		Trout	Phagocytosis ↑, ROI ↑, Antibody →	Sakai et al., 1995f
	NG		American eel	↑ leucocyte binding ability & phagocytic activity. resistence when challenged	Sigel et al., 1983
Ete	Injection	0.5 ml	American eel	↑ phagocytic activity, antibody titres & resistance to challenge	David & Hayasaka, 1984

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection	0.625-1.250/g	Channel catfish	↑ phagocytic activity. No effect on antibody titres. Suppression of resistance when challenged	Stanley et al., 1995
	Injection		Turbot	↑ nonspecific defence, ↑ phagocytosis	Romalde et al., 1999
ET-2	Oral	1.0g/100g feed	Rainbow trout	↑ O^{2-} production. No effect on myeloperoxidase level and survival when challenged.	Siwicki et al., 1994
Evestel	Injection	0.2 ml	Rainbow trout	↑ antibody titre	Cossarini-Dunier, 1985
FCA	Injection	1.2-2.3 x 10^9 cells in FCA	Coho salmon	↑ antibody titre & survival when challenged	Paterson & Fryer, 1974
	Injection & bath	1 mg & 1.0 x 10^{10} cells.ml^{-1} respectively	Rainbow trout	↑ antibody & agglutination titres	Whittington et al., 1994
	Injection		Coho	↑ nonspecific immune system, ↑ resistance to bacterial infection	Olivier et al., 1985
	Injection		Brook	Phagocytosis ↑, ↑ bactericidal activity	Olivier et al., 1986

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection		Trout	↑ disease resistance	Adams et al., 1988
	Injection		Trout	↑ disease resistance	Kajita et al., 1990
	Injection		Yellowtail	↑ disease resistance	Kawakami et al., 1998
	Injection	0.2 ml	Brook trout	↑ survival when challenged	CInjectionriano & Pyle, 1985
FIA	Injection	0.2 ml	Rainbow trout	↑ antibody titre	Cossarini-Dunier, 1985
	Oral	1.5g/100g feed	Rainbow trout	↑ O^{2-} production and myeloperoxidase level, no effect on neutrophil killing activity.	Siwicki et al., 1994
Finnstim	Injection	5, 10, 100ug/fish	Rainbow trout	↑ PFC and humoral antibody	Kitao et al., 1987
FK-565/FK-156	Injection	1 mg/kg	Rainbow trout	↑ Protection against A.salmonicida	Kitao & Yoshida, 1986
	Injection	1 $mg.kg^{-1}$	Rainbow trout	↑ intibody titre & splenic antibody producing cells	Kitao et al., 1987

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection		Trout	Phagocytosis ↑	Kitao & Yoshida, 1986
	In vitro		Trout	Antibody ↑	Kitao et al, 1987
	Injection & immersion	100/g & 100/g.ml^{-1}	Brook trout	↑ survival when challenged	Anderson & Siwicki, 1994
Glucans	Injection	0.1 & 0.2 ml	Atlantic salmon	↑ survival when challenged & antibody titres	Midtlyng et al., 1996
	Injection	2-10 mg/kg	Carp	Activated alternative pathway protection	Yano et al., 1989
	Injection	100 ug-2.5 mg/fish	Atlantic salmon	↑ protection	Robertsen et al., 1990
	Oral	0-1%	Rainbow trout	↑phagocytosis, ↑ ROIs	Volpatti et al., 1998
	Injection/Injection		Trout	Phagocytosis ↑, ROI ↑	Jeney & Anderson, 1993b
Glucan (Sigma)	In vitro		Rainbow trout	↑ respitatory burst activity.	Jang et al., 1995
Glycyrrhizin					
Growth hormone	Injection	0.1 ml/fish	Coho salmon	↑ protection.	Olivier, et al., 1985

Immunostimulant	Administration	Dose	Fish species	Results	Authors
Incomplete Freunds Adjuvant	Injection	0.25 ml	Sockeye salmon	↑ antibody titers	Evelyn, 1971
IFA	Injection	0.1 ml/fish	Brook trout	↑ protection.	Cipriano & Pyle, 1985
	Immersion	1 ug/ml	Rainbow trout	↑ Phagocytic activity ↑PFC	Jeney & Anderson 1993a
ISK	Injection	1 ug	Rainbow trout	↑O^{2-} & phagocytic activity, leucocyte numbers, plaque forming cells, antibody titres & survival when challenged	Anderson & Jeney, 1992
ISK	Immersion	1-5 ug.ml^{-1}	Rainbow trout	↑ O^{2-} & phagocytic activity, leucocyte numbers, plaque forming cells, antibody titres & survival when challenged	Jeney & Anderson, 1993b
ISK	Injection	1 ug/ml	Rainbow trout	↑ neutrophils	Anderson & Jeney, 1993
ISK	Injection	2-10mg/kg BW	Carp	↑ Phagocytosis, ↑ resistance to bacterial infection	Yano et al., 1989
Lentinan	Injection	5-20mg.kg^{-1}	Common carp	↑ leucocyte numbers, phagocytic activity, leucocyte migration, myeloperoxidase activity, lysozyme	Siwicki, 1987

Immunostimulant	Administration	Dose	Fish species	Results	Authors
				level & antibody titre	
Levamisole	Bath	2-20 $mg.dm^{-3}$	Common carp	↑ growth rate	Siwicki & Korwan-Kossakowski, 1988
	Oral	3-8$mg.kg^{-1}$	Common carp	↑ O^2 & phagocytic activity & lysozyme level	Siwicki, 1989
	Injection	0.1-5.0 $mg.kg^{-1}$	Rainbow trout	↑ survival when challenged, phagocytic & natural killer cell activities, CL response. Activation of alternate complement pathway. No effect on bactericidal activity	Kajita et al., 1990
	In vitro		Rainbow trout	↑ O^{2-} & phagocytic activity	Jeney & Anderson, 1993a
	Oral	125-500$mg.kg^{-1}$ feed	Gilthead seabream	↑ phogocytic & respitaroty burst activity in leucocytes, growth, resistance when challenged, lymphokine production & serum complement activity	Mulero et al., 1998a

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection	5 ug.fish^{-1}	Rainbow trout	↑ plaque forming cells, phagocytic index & adherence index	Anderson et al., 1989
	In vitro		Rainbow trout	↑ antibody titre & O^{2-} & phagocytic activity	Siwicki et al., 1990
	Injection	5 ug.fish^{-1}	Rainbow trout	↑ O^{2-} & phagocytic activity, leucocyte numbers & antibody titres	Anderson & Jeney, 1992
	Immersion	5 ug.ml^{-1}	Rainbow trout	↑ O^{2-} & phagocytic activity, leucocyte numbers, antibody titres & survival when challenged. No adjuvant effect on plaque forming cells	Jeney & Anderson, 1993b
	Injection	0.1 ml	Atlantic salmon	↑ survival when challenged.	Midtlyng et al., 1996
	Injection (time studies)	5 ug/fish	Rainbow trout	↑ PFC	Anderson et al., 1989
	Injection	0.5 mg/kg	Rainbow trout	Activated alternative pathway increased phagocytes	Kajita et al., 1990
	Immersion	2 mg/kg	Carp	↑ growth	Siwicki et al., 1988

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection immersion	5 ug/ml	Rainbow trout	↑ protection	Jeney & Anderson, (1993)
	In vitro	5 ug/ml	Rainbow trout	↑ PFC	Anderson et al., 1989
	In vitro	50, 25, 5 ug/ml (in media)	Rainbow trout	↑ neutrophil, phagocytosis and PFC	Siwicki et al., 1990
	Injection		Carp	Phagocytosis ↑	Siwicki 1987
	Oral		Carp	ROI ↑	Siwicki 1989
	In vitro		Trout	Phagocytosis ↑, ROI ↑	Siwicki et al., 1990
	Injection		Trout	Phagocytosis ↑, CL ↑, Complement ↑	Kajita et al, 1990
	Injection		Trout	Phagocytosis ↑, CL ↑	Jeney & Anderson, 1993a
	Injection		Carp	↑ nonspecific defence	Baba et al., 1993

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	In vitro	5-25 mu g/ml	Rainbow trout		Siwicki et al., 1992
	Injection	0.2 ml	Coho salmon	↑ survival when challenged.	Oliver et al., 1985
Levamisole in MFCA	In vitro		Atlantic salmon & Rainbow trout	Stimulation of leucocytes	Reitan & Thuvander, 1991
LPS	In vitro		Atlantic salmon	↑ phagocytic, pinocytic & acid phosphatase activity, intracellular O^{2-} production & stimulatin of macrophages	Dalmo & Seljelid, 1995
	In vitro	1-100 ug	Atlantic salmon	↑ respitatory burst, phagocytic & bactericidal activities	Solem et al., 1995
	In vitro		Catfish	IL1 ↑	Clem et al., 1985
	Injection		Plaice	Macrophage migration ↑	MacArthur et al., 1985
	Injection		Red sea bream	Phagocytosis ↑	Salati et al., 1987
	In vitro		Goldfish	MAF ↑	Neumann et al., 1995
	In vitro		Salmon	Phagocytosis ↑, ROI ↑	Solem et al., 1995

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	In vitro		Salmon	Phagocytosis ↑, ROI ↑	Dalmo & Seljelid, 1995
	In vitro		Atlantic cod	↑ ROI	Steiro et al. 1998
	Injection	0.2 ml	Atlantic salmon	↑survival when challenged	Robertsen et al., 1990
M-glucan	Injection	2.5-5.0 $mg.ml^{-1}$	Atlantic salmon	↑ survival when challenged & antibody titre.	Rorstad et al., 1993
	Injection	200 ul	Atlantic salmon	↑ antibody titre & O^{2-} activity.	Rokstad et al., 1996
Microsilica	Injection	0.2 ml	Atlantic salmon	↑ antibody titre & survival when challenged.	Midtlyng et al., 1996
Mineral oil	Injection	50 ug/fish	Coho salmon	↑ protection	Olivier et al., 1985
Muramyl dInjectioneptide	Injection	0.2 ml	Coho salmon	↑ survival when challenged.	Olivier et al., 1985
Muramyl dInjectioneptide in MFCA	Injection	0.25 mg/kg	Salmon	↑lymphocyte proliferation	Hoel & Lillehaug, 1997
Mycobacterium	Oral		Catfish	ROI ↑	Yoshida et al.,

Immunostimulant	Administration	Dose	Fish species	Results	Authors
chelonae					1995
Oligosaccharide	In vitro		Common carp	↑ phagocytosis, cytotoxic activity	Steinhagen & Hespe, 1997
Parasite lysates	Oral		Yellow tail	Phagocytosis ↑	Itami et al., 1996
Peptidoglucan	In vitro		Atlantic salmon & rainbow trout	Stimulation of leucocytes.	Reitan & Thuvander, 1991
PHA	Injection	0.25-50 $mg.kg^{-1}$	Atlantic salmon	↑ antiboty level and lymphocyte response.	Hoel & Lillehaug, 1997
PGPL-Mc	Injection	50-200 ul	Rainbow trout	No effect on survival when challenged	Horne et al., 1984
Potassium alum	Injection	2.5-5 ug	Rainbow trout	No protection difference.	Horne et al, 1984
	Injection	$0.1mg.g^{-1}$ & oral (0.1 $mg.g^{-1}$	Tilapia	↑ phagocytic activity & survival when challenged.	Park & Jeong, 1996
Polysaccharides	Oral	2-10 $mg.kg^{-1}$	Carp	Range of levels in survival when challenged.	Yano et al., 1991
Prolactin	Oral		Tilapia	Phagocytosis ↑	Park & Jeong , 1996

Immunostimulant	Administration	Dose	Fish species	Results	Authors
PS-K	Injection	1 ug	Rainbow trout	↑ O^{2-} & phagocytic activity, leucocyte numbers, plaque forming cells, antibody titres & survival whcn challenged	Anderson & Jeney, 1992
QAC (Quaternary ammonium compound)	Immersion	1 $ug.ml^{-1}$	Rainbow trout	↑ O^{2-} & phagocytic activity, leucocyte numbers, plaque forming cells, antibody titres & survival when challenged	Jeney & Anderson, 1993b
	Injection immersion	1 ug/ml	Rainbow trout	↑ neutrophil and phagocytic.	Anderson & Jeney, (1993)
	Immersion	10 mg/1	Rainbow trout	↑ bacterial clearance	Grayson et al., 1987
Quil A saponin	Oral		Japanese flounder	↑ survival when challenged, ↑ agglutination titres	Ashida et al., 1999
	Immersion	1650m osmoles	Rainbow trout	↑ antigen uptake	Amend & Fender, 1976
Salt	Immersion	3%	Catfish	↑ antigen uptake	Thune et al., 1984
	Immersion	4.51%	Rainbow trout	↑ antigen uptake	Fender et al., 1978

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Oral	2.7g/100g feed	Rainbow trout	↑ leucocytes, O^{2-}, phagocytic & myeloperoxidase activity, immunoglobulin level & survival when challenged	Siwicki et al., 1994
Saccharomyces cerevisiae	Oral	2.7%	Channel catfish	↑ phogocytic activity	Duncan & Klesius, 1996a
	Injection	10mg/kg BW	Yellow tail	Complement ↑, Phagocytic index ↑, Lysozyme ↑, ↑ disease resistance	Matsuyama et al. 1992
Schizophyllan scleroglucan	Injection		Carp	↑ disease resistance	Yano et al., 1991
	In vitro		Salmon	ROI ↑, Pinocytosis ↑, Acid phosphatase ↑	Sveinbjornsson & Seljelid, 1994
Schizophyllan polyglucose	Oral	0-0.8 $mg.Sekg^{-1}$ diet & 0-240mgvitami	Channel catfish	↑ respitatory burst activity.	Wise et al., 1993a

Immunostimulant	Administration	Dose	Fish species	Results	Authors
		n $E.kg^{-1}$ diet			
Selenium and Vitamin E	Oral		Trout	↑ROI, phagocytosis, killing.	Rumsey et al, 1994
Soy bean protein					
Span tween adjuvant	Oral	2.7%	Channel catfish	↑ chemotaxis, phagocytic activity & antibody levels. No effect on resistance to challenge	Duncan & Klesius, 1996b
Spiralina	Oral		Catfish	CL ↑	Duncan & Klesius, 1996b
	Injection	0.1 ml	Rainbow trout	↑ survival when challenged.	Adams et al., 1988
STA	ISB	0.5 ml	Tilapia	↑ lysozyme activity & number of ROI positive cells from swim bladder, peripheral blood & head kidney.	Chen et al., 1998
Titremax	Immersion	3%	Catfish	↑ antigen uptake	Thune & Plumb, 1984
NaCl	Immersion	4.51%	Rainbow trout	↑ antigen uptake	Fender & Amend, 1978

Immunostimulant	Administration	Dose	Fish species	Results	Authors
NaCl	Oral	1 $g.kg^{-1}$ diet	African catfish	↑ respiratory burst & bactericidal activities.	Yoshida et al., 1993
Vetregard	Injection		Trout	↑ disease resistance	Norqvist et al., 1989
Vibrio bacteria	Injection		Trout	↑ disease resistance	Sakai et al., 1989
	Oral	0.37-15.0$mg.kg^{-1}$ diet	Atlantic salmon	↑ serum antiprotease activity. No effect on respiratory burst, bactericidal, serum lysozyme & serum complement activities, survival when challenged & eicosanoid production.	Thompson et al., 1994
Vitamin A	Oral	0-140mg	Channel catfish	↑ survival when challenged	Durve & Lovell, 1982
Vitamin C	Oral	0-3000 $mg.kg^{-1}$ diet	Channel catfish	↑survival when challenged,antibody levels & complement activity. No effect on phagocytic activity	Li & Lovell, 1985
	Oral	0-2000 $mg.kg^{-1}$ diet	Rainbow trout	↑ growth, survival when challenged & antibody levels	Navarre & Halver, 1989
	Oral	300-2000 $mg.kg^{-1}$ diet	Turbot	↑ serum lysozyme & phagocytic activities	Hardie et al., 1991

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Oral	150-4000 $mg.kg^{-1}$ diet	Rainbow trout	↑ complement (alternataive pathway), CL & phagocytic activities & antibody levels.	Verlhac et al., 1996
Vitamin C and glucan	Oral	7-8000 $mg.kg^{-1}$	Atlantic salmon	↑ phagocytic activity	Hardie et al., 1990
Vitamin E	Oral	0-2500 $mg.kg^{-1}$ diet	Channel catfish	↑ resistance to erythrocyte oxidative haemolysis, phagocytic activity & humoral antibody levels	Wise et al., 1993b
	Injection/oral		Coho	↑ immune response, ↑ disease resistance	Nikl et al., 1991, 1992
VitaSim	Oral/Injection		Chinook	↑ disease resistance	Nikl et al., 1993
	Oral	0.1%	Catfish	Antibody ↑	Ainsworth et al., 1994
	Injection		Salmon	↑ disease resistance	Robertsen et al., 1990
Yeast glucan	Oral		Salmon	↑ disease resistance	Raa et al., 1992
	Injection		Salmon	Complement ↑, Lysozyme ↑	Engstad et al., 1992
	Injection		Catfish	Phagocytosis ↑, Killing ↑, Antibody ↑	Chen & Ainsworth, 1992

Immunostimulant	Administration	Dose	Fish species	Results	Authors
	Injection		Trout	Lysozyme ↑, Killing ↑, O_2 ↑	Jorgensen et al., 1993a
	Injection		Trout	Killing ↑	Jorgensen et al., 1993b
	Injection		Salmon	↑ disease resistance	Rostad et al., 1993
	Injection		Salmon	Lysozyme ↑	Aakre et al., 1994
	Injection		Trout	ROI ↑	Thompson et al., 1995
	Oral		Catfish	ROI ↑	Yoshida et al., 1995
	In vitro		Salmon	Lysozyme ↑, CL ↑	Jorgensen & Robertsen, 1995
	Oral		Turbot	CL ↑, Migration ↑	Baulny et al., 1996
	Oral		Catfish	CL ↑, Migration ↑	Duncan & Klesius, 1996a
	Oral		Trout	↑ CL, ↑Complement	Verlhac et al., 1996

Immunostimulant	Administration	Dose	Fish species	Results	Authors
Yeast glucan + Vitamin C					

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APPENDIX 2

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Findlay V.L., Helders M., Munday B.L. & Gurney R. (1995) Demonstration of resistance to reinfection with *Paramoeba* sp. by Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **18**, 639-642.

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SHORT COMMUNICATION

Demonstration of resistance to reinfection with *Paramoeba* sp. by Atlantic salmon, *Salmo salar* L.

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Amoebic gill disease (AGD), caused by *Paramoeba* sp., is the most serious infectious disease affecting sea-caged Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Tasmania (Munday, Foster, Roubal & Lester 1990). Indeed, this disease, together with the suboptimal osmoregulatory performance of rainbow trout in full sea water, has led to this species being almost exclusively cultured in brackish water. In France, AGD has emerged as a major problem in sea-farmed Atlantic salmon, but it is of lesser importance in rainbow and brown trout, *Salmo trutta* L. (F. Baudin Laurencin, personal communication). Outbreaks in Pacific salmon have been minor and sporadic, and it may be that these species are inherently resistant to the disease. Kent, Sawyer & Hedrick (1988) reported minor outbreaks in coho salmon, *Oncorhynchus kisutch* (Walbaum), in Washington State and California, and C. Anderson (personal communication) has diagnosed occasional outbreaks in chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), held in poor water conditions in New Zealand.

Amoebic gill disease of salmonids in Tasmania is controlled by the use of freshwater baths lasting from 2 to 6 h, and up to three bathings may be undertaken during a normal summer. The long-term success of freshwater baths is dependent on the fish apparently acquiring immunity, and indeed, the third bath is often only given because 10–20% of the fish have relapsed, whereas the remainder appear to be resistant to reinfection (S. Percival, personal communication). Further evidence for the development of a local immunity has been provided by Munday *et al.* (1990), who described the development of lymphoid nodules on the filaments of recovered fish, especially rainbow trout.

Atlantic salmon have been shown to respond to *Paramoeba* antigens, both when immunized parenterally with killed and live organisms, and when subjected to severe natural and experimental infections (Akhlaghi 1994). As well as humoral antibodies, it is possible that local (gill mucus) antibodies may be formed, and as with bacterial gill disease (Lumsden, Ostland, MacPhee, Derksen & Ferguson 1994), these may be protective. The trial described here was designed to test this possibility, as well as provide firm evidence for protective immunity in fish which have previously suffered from AGD.

All experiments were undertaken in 4000-l Rathburn tanks which were attached to individual biofilters to permit recirculation of the water. The water temperature in each tank was controlled by means of a heat exchanger connected to a heat pump with adjustments automatically initiated by a temperature probe connected to an electronic control box. Naive (unexposed) fish were maintained in fresh water and were acclimatized over 7–10 days to sea water when required for challenge experiments.

Salmon were infected with *Paramoeba* sp. by cohabitating them with fish that had AGD, following the advice of L. Searle (personal communication).

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Paramoeba antibodies were quantified using a modification of the enzyme-linked immunosorbent assay (ELISA) described by Bryant, Lester & Whittington (1995). The main variations were:

(1) 3% casein in PBS was used in place of 1% gelatin for blocking;

(2) 0·04% O-phenylenediamine dihydrochloride (OPD) in sodium citrate/phosphate buffer with 0·012% hydrogen peroxide was used in place of ABTS in citrate phosphate buffer with hydrogen peroxide as the indicator; and

(3) sera were absorbed with an equal volume of bacterial antigen (*Xanthomonas maltophilia*) with a protein concentration of 200 μg ml^{-1}.

Sera were used for the detection of humoral antibodies and gill mucus was prepared for ELISA testing according to the method of Lumsden, Ostland, Byrne & Ferguson (1993).

Initially, two replicates of 40 Atlantic salmon smolts of 100 g bodyweight were placed in tanks maintained at 14 °C with 15 Atlantic salmon post-smolts infected with AGD. Within a week, gill lesions, characterized by the development of patches of mucus, were present on some of the naive fish, and by the fourth week, all of these fish had lesions and mortalities had occurred. The surviving fish were transferred to a freshwater tank where they recovered quickly. After a further 4 weeks, the 70 surviving fish were divided into two groups and placed in seawater tanks with equal numbers of naive fish. Fifteen post-smolts with AGD were added to each tank. The severity of the resultant AGD was quantified by counting the number of mucus patches on the first gill arches for both left and right sides (Alexander 1991) every week for 4 weeks. The presence of *Paramoeba* in the lesions was confirmed by microscopic examination of wet preparations of gill scrapings using both bright and dark field illumination.

At the first weekly examination, AGD was present at comparable levels in all groups (Fig. 1). However, thereafter, until the fourth week, the naive fish developed many more lesions and were obviously distressed. The mean number of lesions in the previously exposed fish declined, and indeed, most fish

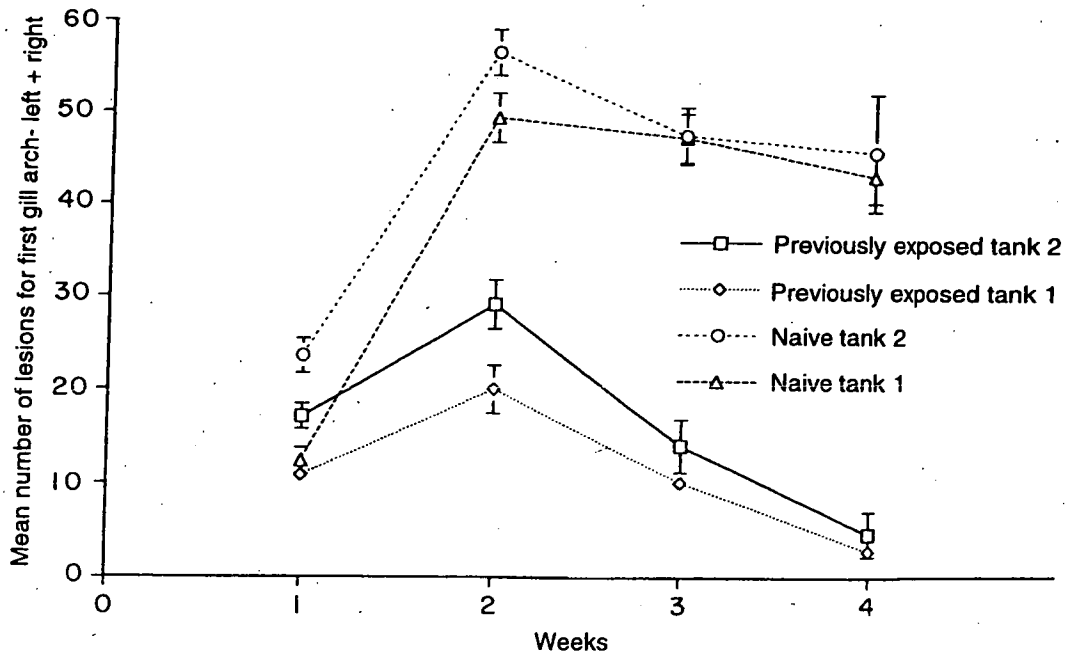

Figure 1. Lesion scores over time for different groups of experimental fish.

completely recovered or were displaying only one or two lesions. However, 11 fish did not recover and accounted for the majority of the total count of mucus patches for this group.

At the end of the trial, all fish were anaesthetized with benzocaine (100 ppm), bled from the caudal vessels and their gills perfused as described in Lumsden *et al.* (1993). One gill was retained for histological examination while the rest were collected for the extraction of mucus for the *Paramoeba* ELISA. The ELISAs were performed using known positive and negative sera as controls. Using the convention that the negative:positive threshold is calculated as the mean of the controls plus three standard deviations (Johnson, Roberts & Munday 1988), it was found that there were no significant ELISA reactions with the mucus samples. However, with the serum samples, it was found that 100% of the donor and freshly infected naive fish were positive as were 68% of the previously infected and re-exposed fish.

This work has confirmed the hypothesis that salmonids which have suffered an attack of AGD are relatively resistant to reinfection. Also, it was noted that, as with field outbreaks, some fish did not develop a strong immunity. Another interesting observation was that the previously exposed fish initially appeared to be as susceptible as the naive fish, but at 2 weeks after re-exposure, they displayed resistance. This phenomenon may be an expression of immunological memory. The absence of demonstrable *Paramoeba* antibodies in gill mucus suggests that surface antibodies are not involved in natural immunity to AGD. However, as fish mucus antibodies are frequently different in composition from serum antibodies and may not be recognized by monoclonals against the latter (Rombout, Taverne, van de Kamp & Taverne-Thiele 1993), this aspect of the immune response to *Paramoeba* sp. must remain unresolved.

As has been demonstrated previously by Akhlagi (1994), infected fish develop a serological response to *Paramoeba* sp. as a result of AGD. Our results are particularly interesting in that the fish with continuous infection (donors and naive fish) had a much higher reactor rate (100%) than those which had been cured of AGD and then re-exposed (68%). As shown in Fig. 1, these latter fish had few active lesions and this suggests that circulating antibody plays little, if any, role in resistance against AGD.

We believe that our observations provide a basis for further investigations, such as the potential for immunostimulants to enhance immunity against AGD and the possible development of vaccines with tropism for the gill epithelium.

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Further studies on acquired resistance to amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L.

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Abstract

Trials were designed to test the efficacy of freshwater treatments for amoebic gill disease (AGD) of Atlantic salmon, *Salmo salar* L., and the effect they had on the acquisition of resistance to reinfection with AGD. The first trial involved fish being given an industry-simulated freshwater bath of 2–3 h duration which simulated treatments given on farms. These fish did not display appreciable resistance to reinfection. The second trial involved four groups of fish which had been infected with and treated for AGD in a number of different ways. Once again the fish that had been infected for the first time and given a single 2–3 h freshwater bath and then re-exposed did not exhibit appreciable resistance to reinfection. In contrast, those fish that had been given a second 2–3 h freshwater bath and those that had been maintained in freshwater for 4 weeks displayed high levels of resistance. There is preliminary evidence to suggest that this resistance could be related to stimulation of the non-specific immune system.

Introduction

As indicated previously by Findlay, Helders, Munday & Gurney (1995) amoebic gill disease (AGD) continues to be the main infectious disease constraining salmonid mariculture in Tasmania. This disease is not confined to Tasmanian waters and has been described in Ireland (Rodger & McArdle 1996), France (F. Baudin Laurencin, personal communication), Washington State and California (Kent, Sawyer & Hedrick 1988) and New Zealand (C. Anderson, personal communication), although to a lesser extent than in Tasmania.

At present, the only successful treatment is the use of a series of freshwater baths given when the fish are experiencing outbreaks of AGD during temperatures in the range of 12–20 °C, and when the fish are held in full salinity water (Munday, Foster, Roubal & Lester 1990).

A series of trials were designed to test a range of treatment possibilities and the effect these would have on the acquisition of resistance to AGD as previously demonstrated by Findlay *et al.* (1995). Of particular interest were the groups of fish that were given industry-simulated treatments, i.e. a freshwater bath of 2–3 h duration followed by a return to full salinity sea water.

Materials and methods

Atlantic salmon weighing between 100 and 200 g were used throughout this study. All fish were naive in relation to AGD, having been maintained in fresh water in 4000 l Rathburn tanks connected to individual biofilters. The water temperature was maintained at 14 °C via an automated temperature probe connected to a heat exchanger and pump. When these naive fish were needed for challenge experiments, they were acclimatized to sea water over a 10 day period. All fish were identified by colour-coded Hallmark® tags which were inserted before the acclimatization period.

Salmon were infected with *Paramoeba* sp. by horizontal transmission after cohabitation with AGD-infected fish as described previously (Findlay *et al.* 1995).

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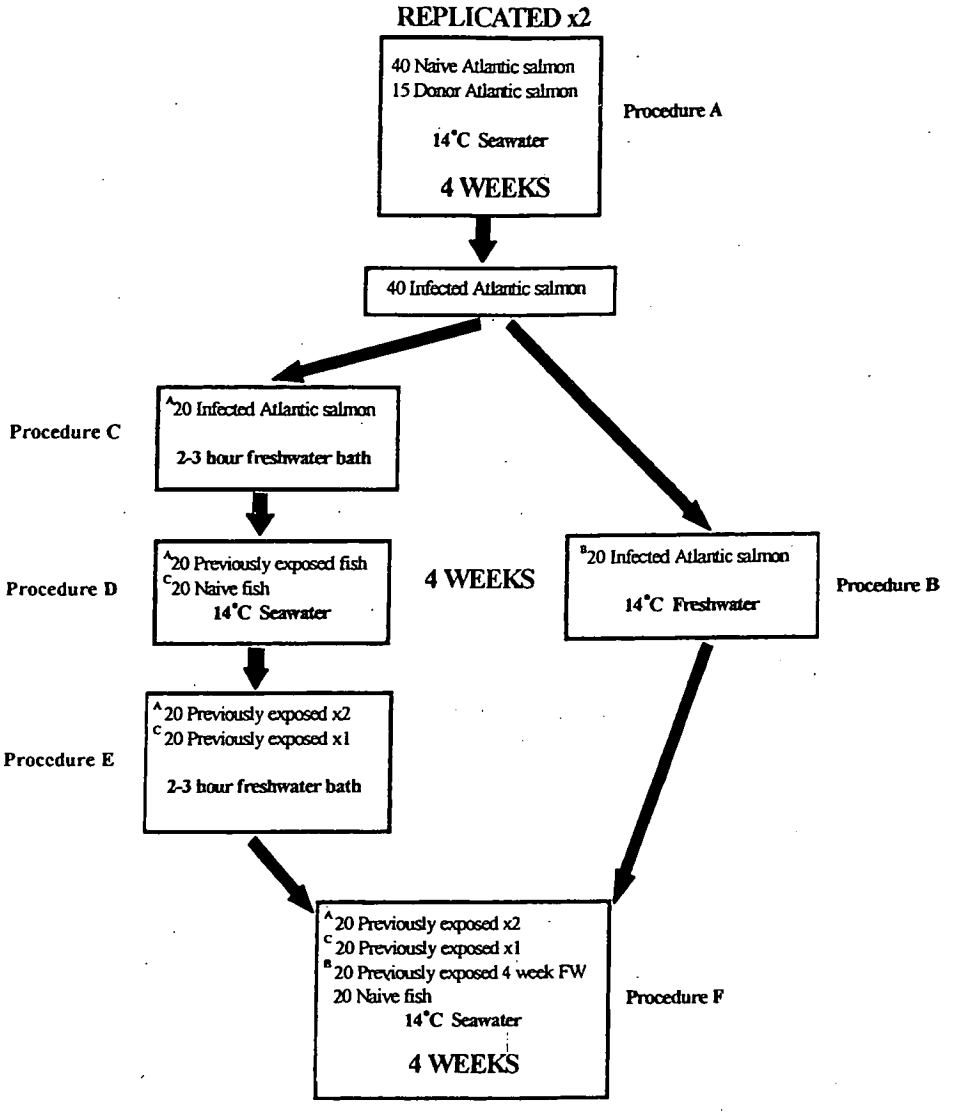

Figure 1 Methods flow chart.

The series of treatments undertaken were relatively complex and can best be understood by reference to the flow chart in Fig. 1.

Initially, two replicates of 40 Atlantic salmon smolts were placed in tanks with 15 Atlantic salmon post-smolts infected with AGD (procedure A). During the first week, gill lesions characterized by mucoid patches were present on a number of the naive fish, and by the end of the fourth week, severe lesions, coupled with the presence of large numbers of *Paramoeba* sp., could be observed on most of the fish. After 4 weeks of infection these previously naïve fish were removed and half were put into fresh water for 4 weeks (procedure B) while the other half were given an industry-simulated freshwater bath of 2 h (procedure C). An industry-simulated bath involves the transfer of fish from sea water to an oxygenated freshwater bath with a maximum salinity of 1‰. As the bath was performed in a separate tank, the opportunity was taken to remove the donor post-smolts, clean the tanks and perform a 100% water exchange. The

treated smolts were then returned to the experimental tanks together with equal numbers of naive fish (procedure D). No infected donor fish were added for the second phase of this challenge, i.e. any recrudescence of AGD would result from residual infection in the treated fish. For 4 weeks the severity of infection was monitored weekly by counting the number of lesions on the first gill arch of both the left and right sides using the method described by Alexander (1991). At the end of the 4 week challenge, all 40 fish were given another industry-simulated bath of 2 h (procedure E) and became the 'previously exposed ×1' and the 'previously exposed ×2' groups in the next challenge.

The second trial involved four groups of fish which had been infected for differing periods of time and treated for AGD in different ways. Into each replicate tank was added 15 naive fish, 15 fish that had been infected once and given a 2 h bath, 15 fish that had been infected twice and given a 2 h bath at the end of each 4 week period, and 15 fish that had been infected once and kept in fresh water for 4 weeks (procedure F). No infected donor fish were added for this challenge.

Weekly, for 4 weeks, the severity of infection was recorded by counting the number of lesions on the left and right sides of the first gill arch as described previously.

Analysis of variance ($P = 0.05$) was performed on all data, using the SAS statistical package. A one-way analysis of variance was performed on data from trial one, while a two-way nested analysis of variance combined with a Tukey's studentized range test was performed on data from trial two.

Results

The results of the first trial are shown in Fig. 2. Fish that had been treated with an industry-simulated, 2 h, freshwater bath displayed little more resistance to reinfection than previously unexposed (naïve) fish. It was not until the middle of the fourth week that some signs of resistance were displayed in the previously exposed fish given a 2 h bath. There was no significant difference between the previously exposed groups and the naïve groups for the first 3 weeks ($P = 0.679$, d.f. = 1; $P = 0.858$, d.f. = 1; and $P = 0.167$, d.f. = 1, respectively). However, by week 4, the previously exposed fish displayed significantly fewer lesions than their naïve counterparts ($P = 0.008$, d.f. = 1).

Figure 3 illustrates the changes in lesion numbers recorded for the fish in the second trial. It should be noted that it was necessary to truncate observations of the naive and 'previously exposed ×1' treatment because of the mortalities due to AGD in these fish.

In contrast, the group of fish that had experienced two waves of infection, and had been treated with industry-simulated freshwater baths of 2 h each, displayed only a moderate level of disease 1 week after challenge. There was a significant difference between treatments for week 1 ($P = 0.0001$, d.f. = 3). The results of the Tukey's test showed that there was a significant difference between all treatments with the exception of those that had been 'previously exposed ×2' and those that had been kept in fresh water for 4 weeks. The significant difference between treatments remained for weeks 2 and 3 ($P = 0.0001$, d.f. = 3 and $P = 0.0001$, d.f. = 3, respectively). The Tukey's test grouped the fish that had been given the 4 week freshwater bath with those given two rounds of industry-simulated freshwater baths (group 1) and the 'previously exposed ×1' fish with the naïve fish (group 2). The fish grouped in the first instance displayed significantly fewer lesions than those fish grouped in the second. This difference remained consistent in week 3. In week 4 no significant difference was found between treatments ($P = 0.542$, d.f. = 1). This, however, is purely numeric because observations for the 'previously exposed ×1' treatment and the naïve treatment were truncated due to mortalities. It is obvious from Fig. 3 that the greatest potential difference between treatments existed in week 4. In fish that had been given two rounds of freshwater bathing, only a small number displayed lesions by the end of the fourth week. For those fish that had been given a 4 week freshwater bath, only a small number ever displayed lesions, and even then at a low level. In both these latter groups of fish, it is interesting to note that a small number of fish accounted for the majority of lesions. This is in contrast to the 'previously exposed ×1' and naïve treatments in which no or few fish survived past the third week. In fact, the remaining fish of these groups had to be rebathed to abate the high mortality rate.

Discussion

It is now well established that fish that have been previously exposed to AGD show resistance to reinfection (Findlay *et al.* 1995). In that earlier

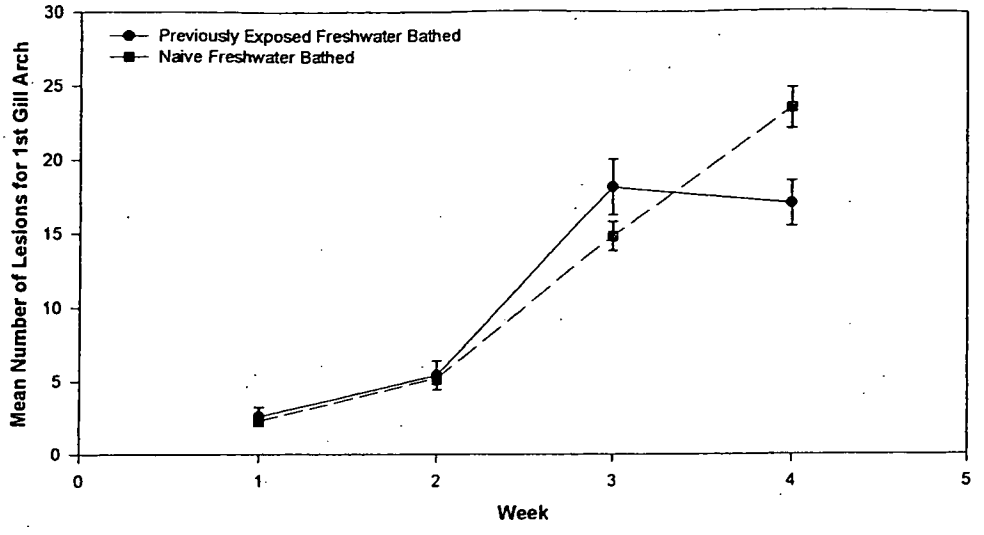

Figure 2 Changes in AGD lesion numbers (first trial).

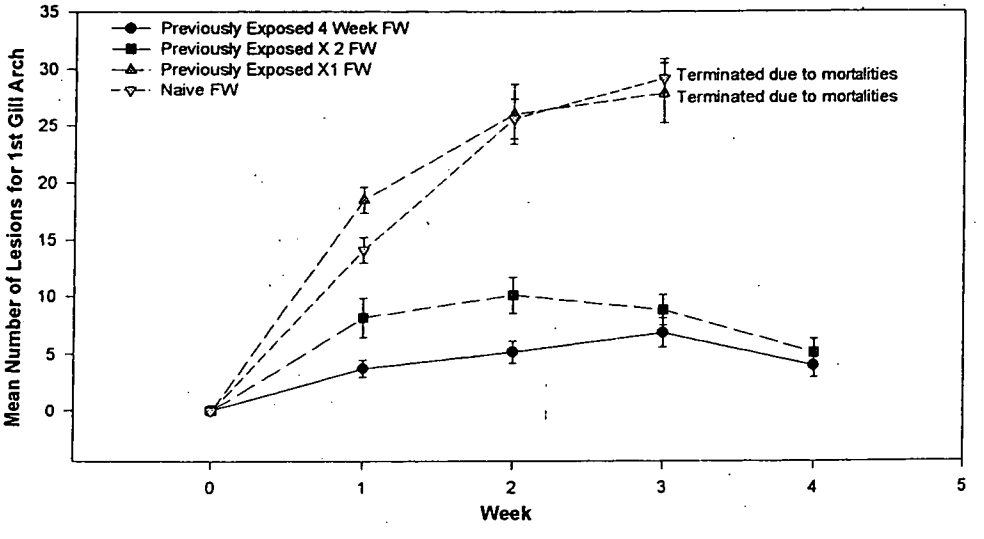

Figure 3 Changes in AGD lesion numbers (second trial).

paper, it was shown that fish that had been exposed to AGD, kept in fresh water for 4 weeks and then re-challenged displayed a high level of resistance to reinfection. It is interesting that this same level of resistance is not reached when fish are given a single, industry-simulated freshwater bath of 2–3 h. Even the small decrease in lesion numbers in the fourth week after the freshwater bath is artificial due to the coalescence of lesions when fish exhibit such high numbers, as well as the death of the most severely affected fish which were carrying the highest number of lesions. Thus, it appears that the fish which are bathed for 2–3 h for the first time and then re-exposed to *Paramoeba* sp. do not develop appreciable signs of resistance to reinfection. In fact, their ability to cope with *Paramoeba* sp. challenge seems little different from that of fish which are experiencing their first wave of infection. In contrast, fish which have been given two industry-simulated baths display a high level of resistance to reinfection and have an average of fewer than five lesions per first gill arch at the termination of the trial. It is notable that by weeks 3 and 4 after treatment, there was no difference between this group of fish and

those that had remained in fresh water for 4 weeks. The initial difference between these latter two groups of fish during weeks 1 and 2 may be explained by the fact that fish which are given a 2–3 h bath probably still maintain low levels of amoebae on their gills after the bath, while fish which have been held in fresh water for 4 weeks are completely cured before being re-challenged. This point is of particular interest because in the trials which involve fish being given an industry-simulated bath no donor fish are needed to re-establish infections, thus indicating some carry-over by treated fish. This is in contrast to the trials in which fish are held in fresh water for 4 weeks. This point has led us to believe that there may not necessarily be a reservoir of infection in the immediate environment, but rather there are enough viable infective stages left on the fish after a 2–3 h bath that reinfection is immediately facilitated once the fish are returned to sea water.

As we have previously shown in Findlay *et al.* (1995), circulating antibody appears to play little, if any, role in resistance to amoebic gill disease. The resistance demonstrated may be due to stimulation of the non-specific immune system by AGD infection. On this basis, further trials involving the assessment of the non-specific immune system and the use of immunostimulants to enhance this process have been initiated.

Acknowledgments

We wish to thank the Cooperative Research Centre for Aquaculture for providing monetary support for research and a scholarship for the senior author. Thanks are owed to James Findlay for invaluable assistance in many areas of this research and to Aquatas and Salmon Enterprises of Tasmania for support in kind.

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Evaluation of levamisole as a treatment for amoebic gill disease of Atlantic salmon, *Salmo salar* L.

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Abstract

Levamisole, a known T-cell stimulator and immunomodulator in mammals, has been demonstrated to enhance resistance to amoebic gill disease in Atlantic salmon, *Salmo salar* L. When used in fresh water baths, dose rates of 1.25, 2.5 and 5 ppm levamisole stimulated resistance to reinfection with *Paramoeba* sp. that was evident from 2–3 weeks post-treatment. It is proposed that this response is related to enhancement of the non-specific immune system.

Introduction

Amoebic gill disease (AGD) has been diagnosed in Atlantic salmon, *Salmo salar* L., in Tasmania (Munday 1985), Ireland (Palmer, Carson, Ruttledge, Drinan & Wagner 1997), France (Findlay & Munday 1998), Chile (D. Groman, personal communication) and the Pacific Northwest of North America (M. Sheppard, personal communication). It is the most important infectious disease affecting sea-caged salmon in Tasmania (Findlay & Munday 1998) and has emerged as a significant problem in Ireland (R. Palmer, personal communication). At present the only successful treatment is a series of fresh water baths of 2–3 h duration given when the fish are experiencing outbreaks of AGD (Munday, Foster, Roubal & Lester 1990). Many attempts to find a chemotherapeutic agent have been fruitless (Alexander 1991; Cameron 1994), but Howard & Carson (1994) have shown that a number of compounds, including levamisole, are capable of inhibiting the growth of the causative agent, *Paramoeba* sp. *in vitro*. Also, Findlay, Helders, Munday & Gurney (1995) found that some fish did not develop useful resistance to reinfection with *Paramoeba* sp. after acquiring AGD and being treated with a fresh water bath, suggesting immunological impairment. As such immunologically-impaired animals often respond to levamisole (Symoens & Rosenthal 1977), this was another reason to assess the utility of this compound for treating AGD in Atlantic salmon.

The studies reported here were designed to test the efficacy of levamisole as an immunomodulator in the treatment of AGD of Atlantic salmon.

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Materials and methods

Atlantic salmon of 100–200 g body weight (BW) were used throughout this study. Fish were not previously exposed to AGD, having been maintained in fresh water in 4000 L Rathburn tanks connected to individual biofilters. The water temperature was maintained at 14 °C via an automated temperature probe connected to a heat exchanger and pump.

The study was based on the experimental design described by Findlay & Munday (1998). In brief, fish which had previously experienced no, one or two episodes of AGD were given a variety of fresh water bath treatments with different levels of levamisole, and then re-exposed to infection in the form of cohabitation with clinically diseased fish.

Details of the three individual trials are given in Table 1. For 4 weeks, the severity of infection was monitored weekly by counting the number of mucoid patches, typical of AGD in Atlantic salmon, on the first gill arch of both the left and right sides using the method described by Alexander (1991). This has been verified as an appropriate method for monitoring experimental AGD (Findlay *et al.* 1995; Findlay & Munday 1998; Zilberg & Munday, unpublished)

Two way analysis of variance (ANOVA) and Tukey's post hoc tests were performed to compare the number of gill lesions in the different treatments in each tank, and between the replicate tanks (Sigma Stat, 1992–1994, Jandel Corporation). Data were considered significant at a 5% level ($P < 0.05$).

Results

Trial One

Lesion numbers due to AGD were significantly reduced among levamisole-treated salmon experiencing their second wave of infection, when compared to fish treated with fresh water only ($P < 0.05$; Fig. 1). This was particularly apparent at 3 and 4 weeks post-exposure to the disease. Lesions on levamisole-treated salmon experiencing their first wave of infection were variably significantly lower than those on the fresh water treated fish ($P < 0.05$; Fig. 2).

Trial Two

It was noted that the continual passage of AGD through susceptible fish apparently raised the virulence of *Paramoeba* sp. By the end of this trial (Trial Two) the effects of this increased virulence were evident. While the pattern of infection remained similar to that of Trial One, the magnitude of infection was greater, thus affecting the outcome compared with Trial One. Because of the mortalities that occurred in naive fish and fish that had been exposed to AGD on one occasion, but without levamisole treatment, these two groups were prematurely withdrawn from the trial by 4 weeks post-exposure. Levamisole treatment appeared to be of assistance to naïve fish and those that had experienced one wave of infection and had been immediately returned to sea water, significantly reducing the number of gill lesions at weeks 2–4 post-exposure ($P < 0.05$; Fig. 2). Levamisole significantly reduced the number of patches in fish that had experienced two waves of infection at weeks 1 and 2 post-exposure ($P < 0.05$), but not later in the trial ($P > 0.05$; Fig. 2). Also, such treatment did not affect the outcome for fish that had been returned to fresh water for 4 weeks after initial infection.

Trial Three

In Trial Three, groups of fish were treated with different concentrations of levamisole. There were

Table 1 Summary data of experimental procedures for levamisole supplementation in fresh water bath treatments (Trials 1, 2 and 3)

Trial no.	Treatment	Levamisole dose (mg/L)	No. of fish in replicate tank 1	No. of fish in replicate tank 2
1	PE × 1Lev	5.0	18	18
1	PE × 1FW	0	18	18
1	Naïve Lev	5.0	18	18
1	Naïve FW	0	18	18
2	PE 4FW Lev	5.0	15	15
2	PE 4FW FW	0	15	15
2	PE × 2 Lev	5.0	15	15
2	PE × 2 FW	0	15	15
2	PE × 1 Lev	5.0	15	15
2	PE × 1FW	0	15	15
2	Naïve Lev	5.0	15	15
2	Naïve FW	0	15	15
3	PE × 1 Lev	5.0	17	19
3	PE × 1 Lev	2.5	17	14
3	PE × 1 Lev	1.25	18	15
3	PE × 1 FW	0	19	20
3	Naïve FW	0	17	17

PE = previously exposed, FW = 2–3 h fresh water bath only, Lev = 2–3 h fresh water bath + levamisole, 4FW = 4 weeks in fresh water before re-exposure.

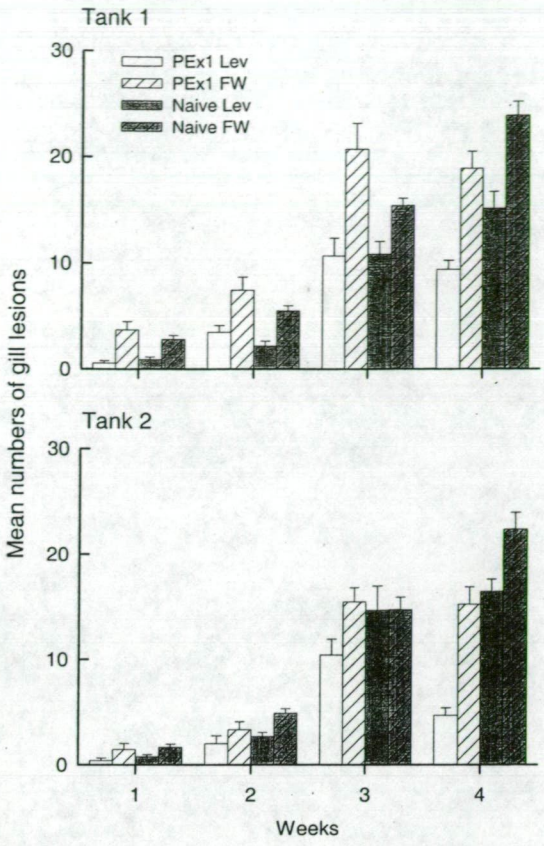

Figure 1 Mean number of gill lesions on AGD-infected fish experiencing their first (naïve) or second (PE × 1) waves of infection. Fish were treated with fresh water bath with or without 5 ppm levamisole, and equally divided between two 4000 L tanks ($n = 18$ per replicate tank). PE = previously exposed, FW = fresh water bath only, Lev = fresh water bath + levamisole.

no significant differences between lesion numbers for any of the groups treated with levamisole throughout the experiment ($P > 0.05$; Fig. 4). However, the groups given a fresh water bath only had significantly more lesions at weeks 3 and 4 post–exposure ($P < 0.001$; Fig. 3). Fish that were previously exposed and treated with fresh water only had fewer lesions compared to naive fish on weeks 3 and 4 in tank 1 ($P < 0.05$) and on week four in tank 2 ($P < 0.001$; Fig. 3).

Discussion

In regard to the beneficial effects of low concentrations of levamisole added to a fresh water bath at the rate of 1.25–5 ppm, there is ample evidence for low levels of levamisole acting as immunostimulants in fish, including Atlantic salmon (Siwicki 1987, 1989; Kajita, Sakai, Atsuta & Kobayashi 1990; Jeney, Galeotti & Volpatti 1994; Mulero, Esteban, Munoz & Meseguer 1998; Findlay & Munday 2000). The fact that levamisole is a T-cell stimulant is particularly relevant because Lin, Ellis, Davidson & Secombes (1999) have shown that leucocytes isolated from rainbow trout gills are predominantly T-cells. As a 5 ppm levamisole bath reduces the antibody response in salmon (Morrison 1998) and as there is no apparent relationship between detectable antibodies against *Paramoeba* sp. and resistance to AGD (Findlay *et al.*, 1995; Akhlaghi, Munday, Rough & Whittington 1996), this potential aspect of the drugs immunomodulatory repertoire is not likely to be relevant. Also, *in vitro* experiments have shown no deleterious effects of anti-*Paramoeba* antibody on *Paramoeba* sp. from Atlantic salmon gills (Zilberg & Munday, unpublished).

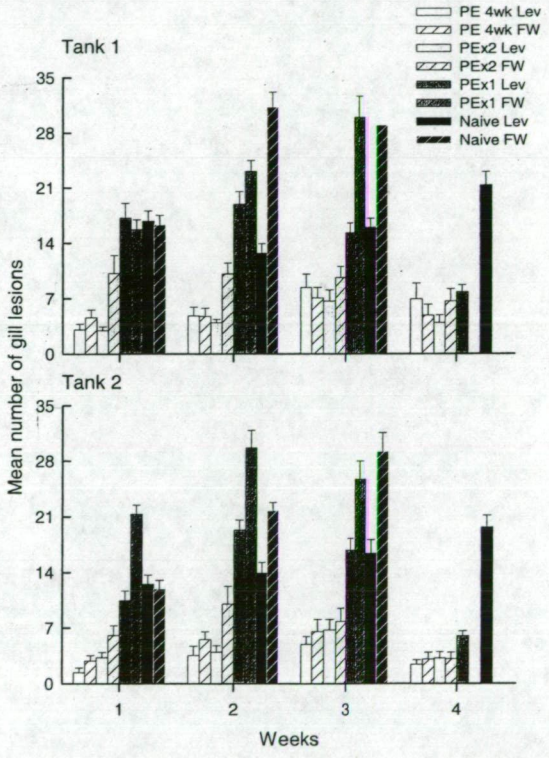

Figure 2 Mean number of gill lesions on AGD-infected fish experiencing their first (naïve), second (PE × 1) or third (PE × 2) waves of infection, and a group of fish that was kept in fresh water for 4 weeks following their first wave of infection (PE 4 weeks). Each one of these groups was treated with a fresh water bath with or without 5 ppm levamisole, and equally divided between two 4000 L tanks ($n = 15$ per replicate tank). PE = previously exposed, FW = fresh water bath only, Lev = fresh water bath + levamisole, 4FW = 4 weeks in fresh water before re-exposure.

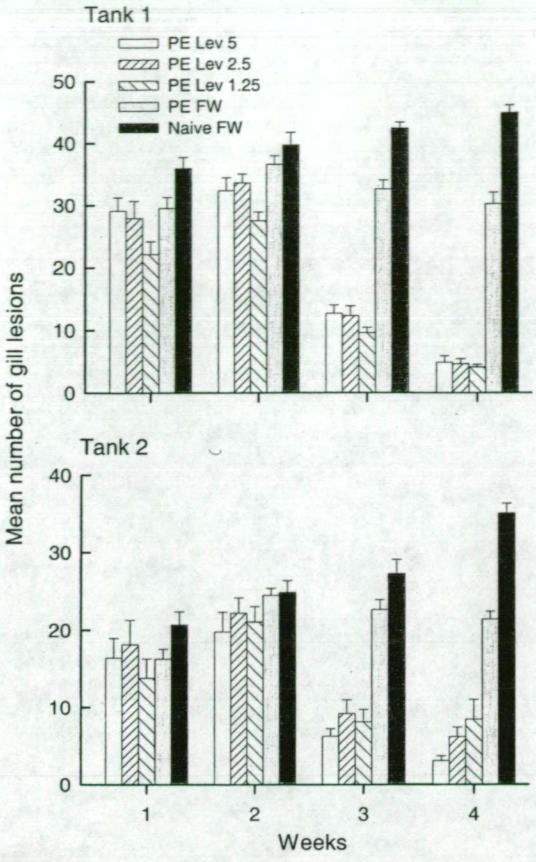

Figure 3 Mean number of gill lesions on AGD-infected fish experiencing their first (naïve) or second (PE × 1) waves of infection. Fish experiencing their second wave of infection were treated with a fresh water bath with or without levamisole, at concentrations of 1.25, 2.5 and 5 ppm. Naïve fish were treated only with a fresh water bath. Each group was equally divided between two 4000 L tanks ($n = 14–20$ per replicate tank, see Table 1). PE = previously exposed, FW = fresh water bath only, Lev = fresh water bath + levamisole.

Much of the work reported here used the method of Alexander (1991) to quantify AGD infection. The validity of this method for field trials has since been questioned by Clark & Nowak (1999). However the authors' experience is that the use of counts of gill patches is a valid method for experimental AGD, although care is needed to ensure that standardized methods are used throughout individual trials.

While there were significant decreases in lesion numbers in most of the groups of fish treated with levamisole, it was those fish experiencing their second wave of infection (i.e. fish which had previously been infected and given a fresh water bath before being re-exposed) that benefited most from the treatments. Incidental mortality data collected from the levamisole-treated fish support these findings (Zilberg, Findlay, Girling & Munday 2000). Possibly this is the result of combined responses to both the infection and the immunomodulator. Fish that had experienced even more waves of infection presumably develop a high level of non-specific immunity (Findlay *et al.* 1995; Akhlaghi *et al.* 1996; Findlay & Munday 1998) and the synergistic effect of levamisole is not so apparent. It is notable that fish which have been infected and allowed to recover fully in fresh water for 4 weeks, display significant resistance to reinfection, whether or not they are treated with levamisole. It appears that stimulation of the non-specific immune system following one wave of AGD, coupled with the fact that the fish's gills would have recovered completely from the infection, ensures that these fish are almost completely refractory to reinfection.

It appears that re-establishment and development of AGD is the result of interplay between immune responses (especially the non-specific immune response), gill health, number and virulence of *Paramoeba* and environmental conditions (especially salinity and temperature). In this context, it has been reported that levamisole significantly enhances the healing process in mammals (Symoens & Rosenthal 1977). This enhanced healing was attributed to the increase of neutrophil and macrophage migration to the damaged area, and it is reasonable to assume that a similar mechanism may exist in teleosts.

To put the various responses to levamisole given as a bath treatment in context, it is suggested that the factors involved are as follows:

1. Fish infected for the first time and given a 2 h fresh water bath only will develop a moderate increase in their non-specific immunity and will have mucus and amoebae removed from their gills. However, the lesions of gill hyperplasia and inflammation will remain to attract amoebae (Nowak & Munday 1994) and some amoebae may survive within cystic lesions present in the gills (Munday *et al.* 1990).

2. Fish infected for the first time and given a 2 h fresh water bath containing levamisole will experience a very much enhanced non-specific immune response and, therefore, there will more likely be resolution, rather than persistence, of infection and resultant lesions.

3. In fish that have been previously exposed on two occasions and given two industry-simulated baths lesions are still present, but the nonspecific immune response has been augmented to a sufficient level to allow recovery. In this instance levamisole provides only a temporary advantage of a slightly higher resistance to reinfection in the early weeks of exposure.

4. Fish that have been infected for the first time and allowed to recover in fresh water for 4 weeks have gills that are in excellent condition when re-exposed, so while their immune response may not remain at a high level, the condition of the gills compensates for this.

The concentrations of levamisole used in Trials One, Two and Three were less than that shown by Howard & Carson (1994) to inhibit the growth of *Paramoeba* sp. *in vitro* ($\geq$ 10 ppm). Therefore, it is reasonable to suggest that the effects reported here are entirely due to the drugs' immunomodulatory activity.

With the increasing need for the use of therapeutants in aquaculture, it is very important that potential chemicals be chosen carefully so as to minimize the accumulation of chemicals in food for human consumption and the effect on the environment. In this context, since its introduction levamisole has been used extensively and safely in veterinary and human medicine (Symoens & Rosenthal 1977; Arundel 1985; Anon. 1991). Levamisole is a simple chemical that is rapidly adsorbed and excreted. Peak blood levels in farm animals are reached 2–3 h following oral dosage and concentrations then decrease, little being present 20 h after oral dosage. In farm animals it is almost totally excreted in the urine and bronchial mucus. In man, levamisole has a plasma half-life of about 4 h, is extensively metabolized in the liver and is virtually eliminated from the body within 2 days (Symoens & Rosenthal 1977). The half-life in eels at 19–23 °C is remarkably similar at 3.99 h (Blanc, Loussouarn & Pinault 1991).

Levamisole is quite stable in acid aqueous media but hydrolyzes in alkaline solutions such as sea water (Symoens & Rosenthal 1977) and, therefore, is particularly suitable for use in fresh water baths, which are subsequently released into the sea.

In conclusion, the present challenge experiments clearly demonstrate that levamisole, when given in a fresh water bath at a range of concentrations, can be therapeutic for fish with AGD.

Acknowledgements

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The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L.

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Abstract

Sea water-adapted Atlantic salmon, *Salmo salar* L., were given a 2-h bath in a 2.5 mg L^{-1} levamisole (as levamisole hydrochloride) solution in fresh-water. Following bathing, the fish were held in full salinity sea water for 2 weeks before being subjected to a number of immunological assays. Heightened activity of the nonspecific defence system was demonstrated by increases in phagocytic index, phagocytic capacity and phagocytic activity, increased levels of the reactive oxygen intermediate, superoxide anion, and an increased lytic activity of both the mucus and the serum. These results indicate that levamisole is effective in augmenting parts of the nonspecific defence system of Atlantic salmon. This is the first record of the use and efficacy of levamisole as an immunomodulator in Atlantic salmon.

Introduction

Nonspecific defence mechanisms are important to all vertebrates. Fish, however, depend more heavily on these nonspecific mechanisms than do mammals and this is especially true of cold water species (Avtalion 1981). Furthermore, in those instances where pathogens infect sites that are relatively insulated from the specific humoral immune system, such as mucous surfaces, there seem to be few available options for treatment or prophylaxis, other than direct chemical therapeutics, or modulation of the nonspecific defence system.

In the last decade there has been increasing interest in the modulation of the nonspecific immune system of fish as both a treatment and prophylactic measure against disease. A number of substances, including levamisole, have been shown to heighten the nonspecific defence system capacity.

Levamisole is a levo-isomer of tetramisole. The compound was first introduced in 1966 as a broad spectrum anthelmintic (Theinpont, Vanparijs, Raeymaekers, Vandenberk, Demoen, Allewijn, Marsboom, Neinmegeers, Schellekens & Janssen 1966) and has since been used extensively and safely in veterinary and human medicine. Accumulated evidence has strongly suggested that levamisole treatment leads to an enhanced state of resistance to various kinds of infections (Symoens & Rosenthal 1977; Jeney, Galeotti & Volpatti 1994; Mulero, Esteban, Munoz & Meseguer 1998). In this study augmentation of the nonspecific immune systems including lysozyme, reactive oxygen species and phagocytic ability in Atlantic salmon following levamisole treatment is reported for the first time.

Studies have shown that bath treatment of Atlantic salmon with levamisole resulted in increased resistance to amoebic gill disease (AGD) (Findlay, Zilberg & Munday, 2000). Levamisole has been demonstrated to modulate both the specific and nonspecific defence systems but, as has been demonstrated in previous studies (Findlay, Helders, Munday & Gurney 1995), there is no correlation between detectable antibody against the *Paramoeba* sp. causing AGD and resistance to disease. There-

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fore, it has been hypothesized that the resistance levamisole affords Atlantic salmon is mediated via the nonspecific defence system. This hypothesis was the stimulus for the study described here.

Materials and Methods

Fish

All experimental fish were post-smolt Atlantic salmon, *Salmo salar* L., weighing between 100 and 200 g. Fish were maintained in full salinity sea water in 4000 L Rathburn tanks connected to individual biofilters. The water temperature was maintained at 14 °C via an automated temperature probe connected to a heat exchanger and pump. All fish were identified by colour coded Hallmark® tags which were inserted prior to each experiment.

Of the fish in each group, half were bathed in a fresh water bath with levamisole added at a rate of 2.5 mg L^{-1} (treatment group) and the other half in a plain fresh water bath (control group). The fresh water bath mimics that used by industry to treat AGD and the dose rate of 2.5 mg L^{-1} of levamisole has previously been demonstrated to increase resistance of Atlantic salmon to AGD (Findlay *et al.* 2000). Fish were killed 14 days post-treatment as this has previously been shown by Findlay *et al.* (2000) to be the time at which recovery from AGD becomes most consistently apparent in levamisole treated fish.

Techniques

Collection of serum and mucus

To collect mucus samples the gills were first perfused. With head elevated, the tail was severed at the peduncle region and then the heart was exposed. The gills were perfused with heparinized saline by direct puncture of the bulbus arteriosus or ventricle. Depending on the size of the fish, complete perfusion occurred after about 30 sec and after 20–30 mL of saline had been injected. Perfusion was judged as being complete when the gills were blanched white and the fluid from the caudal vessels was clear. The gills were then excised and the mucus removed with the blunt side of a plastic knife. Any contaminating detritus was removed by centrifugation (1500 × *g* for 10 min). The samples were examined for evidence of contamination by blood.

Serum samples were collected by bleeding from the caudal vein. The blood was allowed to clot at room temperature for 1 h, kept for 8 h at 4 °C and then centrifuged at 1500 × *g* for 10 min to obtain the serum. Serum and mucus samples were stored at −80°C or used immediately.

Preparation of head kidney cell suspension

The head kidney was removed and placed on a stainless steel mesh sieve (0.3 mm mesh) in a Petri dish containing 5 mL of PBS. The kidney tissue was pushed through the mesh and the resulting homogenate passed through a loosely-packed, glass wool syringe column to remove tissue fragments, cellular debris and red blood cells. The suspension was allowed to settle for 15 min and then gently layered upon 2 mL of Histopaque 1077 (Sigma). The layered cell suspension was centrifuged for 15 min at 400 × *g*.

Following centrifugation, the white blood cell interface layer was removed and washed in 3 mL PBS (3 min at 200 × *g*). The supernatant was decanted and the pellet resuspended in 0.5 mL of PBS. The solution was vortex mixed and a drop placed on a glass slide and covered with a coverslip. Cell viability ($\geq$ 95%) was confirmed using trypan blue exclusion.

Assays

Lysozyme assay

A turbidometric assay utilizing *Micrococcus lysodeikticus* lyophilized cells (Sigma) was used to determine lysozyme concentrations in the serum and mucus collected from Atlantic salmon. Eight samples were used in each assay. The method was a modification of that used by Sankaran & Gurnani (1972) who reported differences in the optimal pH and buffer molarities according to whether the fish were from fresh water or sea water. Therefore, a series of assays was conducted with pooled mucus and serum samples to optimize the test for use with Atlantic salmon maintained in sea water. While it is recognized that different buffers may further optimize results it was important to maintain some comparability so the same buffer (0.04 M phosphate buffer) was used for all assays.

M. lysodeikticus was suspended in phosphate buffer at a concentration of 0.25 mg mL^{-1}. Two hundred microlitres of serum or mucus, diluted

with an equal volume of PBS, were added to 1.3 mL of the substrate solution at 25 °C and the optical density at 450 nm read immediately. After 30 min incubation in a humidified environment at 25 °C the optical density was measured once again. Lyophilized hen egg white lysozyme (HEWL) was used to develop a standard curve.

Serum and mucus lysozyme values are expressed as μg mL^{-1} equivalent of hen egg white activity and were derived using the equation for the second order polynomial regression line.

Phagocytosis assay

The ability of fish phagocytes to ingest particles is usually demonstrated with an *in vitro* phagocytosis assay (Duda 1996). These tests are expensive and labour intensive. In the present study, an *in vivo* phagocytosis assay that is very cheap to run and extremely robust (Duda 1996) was used.

Two groups of fish were used in this assay; the first group was treated with levamisole and the second acted as a control group and were bathed in fresh water only. There were 20 fish per group.

The phagocytic abilities of head kidney cells were examined as follows. Suspensions of cells of the yeast *Saccaromyces cerevisiae* 10^8 mL^{-1} were prepared in phosphate buffered saline (PBS) (pH 7.4). The equivalent amount of 0.8% congo red was added to the yeast suspension before it was autoclaved at 121 °C for 20 min (the yeast cells may then be refrigerated for up to 72 h). Before use, the yeast cells were washed five times in sterile PBS for 3 min at 300 × *g* and resuspended in the initial volume of PBS.

Fish were anaesthetized with 40 mg L^{-1} benzocaine (10% wv-1 ethyl-4-aminobenzoate in acetone) to Stage III or surgical procedure anaesthesia as described by Ross & Ross (1984), weighed and injected with 0.1 mL per 100 g body weight of the yeast solution via the caudal vein. The fish were revived and held in 400 L plastic tanks supplied with oxygen for 2 h. After the holding period the fish were anaesthetized once again and bled by severing the caudal peduncle.

Head kidney cells were collected using the method described above and the phagocytic cells, including neutrophils and macrophages, were counted until 100 cells that had consumed yeast had been recorded. The number of yeast cells in each phagocyte was counted and the phagocytic index, phagocytic capacity and phagocytic activity calculated as follows: phagocytic index (PI) equals the total number of yeast cells consumed, divided by the number of consuming phagocytes. The phagocytic capacity (PC) equals the total number of phagocytes containing a given number of yeast cells divided by the total number of phagocytes containing any yeast. Phagocytic activity (PA) is expressed as the number of phagocytizing cells divided by the total number of phagocytes counted.

Reactive oxygen intermediate — superoxide anion assay

Superoxide produced by the macrophages isolated from the head kidney was measured by the reduction of ferricytochrome C as described by Secombes, Chung & Jefferies (1988) and Zelikoff, Wang, Islam, Twerdok, Curry, Beaman & Flescher (1996). The specificity of the reaction was demonstrated by preventing the reduction of ferricytochrome C with exogenous superoxide dismutase (SOD) which dismutates any O_2^- generated to hydrogen peroxide.

The amount of O_2^- produced in the respiratory burst was quantified by comparing cells taken from an individual fish using four different reaction mixtures. Each of these four mixtures contained 500 μL of ferricytochrome C (Sigma) (final concentration of 2 mg mL^{-1} prepared in supplemented fish physiological saline) to which had been added 10^6 kidney cells (in a total volume of 250 μL of supplemented Leibovitz medium). The first two reaction mixtures measured basal levels of O_2^- so no membrane stimulant was added to these mixtures. Exogenous SOD [125 μL at a final concentration of 37.5 μg mL^{-1} prepared in Hanks buffered salt solution (HBSS)] was added to one of these mixtures so any basal level O_2^- was inhibited. The second two reaction mixtures mimicked the first, with the addition of 20 μL of the soluble stimulant phorbal myristate acetate (PMA) (Sigma) (at a final concentration of 2.0 μg mL^{-1}, prepared in dimethylsulphoxide, working solution of 100 μg mL^{-1} prepared in HBSS). An additional tube that contained all of the above-mentioned reagents, but without cells, acted as the reaction blank. Fish physiological saline was added to all the mixtures to bring the final volume up to 1 mL.

Each of the mixtures was vortexed for 30 sec before 200 µL aliquots were placed in triplicate in 96-well microtitre plates. The absorbance was measured at 550 nm every 10 min for 2 h and again at 24 h. Between readings the plates were placed in a humidified incubator at 30 °C.

Change in absorbance was calculated by first subtracting the mean of the blank wells from all other wells and then subtracting the absorbance of the wells containing SOD from that of the non-SOD-containing wells. The results were expressed by converting the optical density readings to nmol O_2^- per 10^5 cells by multiplying with the correction factor of 15.87 as given by Pick & Mizel (1981).

Haematocrit and leucocrit

Fish were anaesthetized as previously described and a blood sample taken from the caudal vein. Heparinized haematocrit capillary tubes (Chase Instrument Corporation, Glens Falls, NY, USA) were filled to the red line. Duplicate samples were taken from each fish. The tubes were then centrifuged for 1 min in a haematocrit centrifuge. The percentage erythrocyte (haematocrit) and leucocyte (leucocrit) volume was calculated by overlaying the tubes on a sliding scale haematocrit reader.

Statistical analysis

The regression for the HEWL standard curve was undertaken using the statistical package of Microsoft Excel 97. One way and nested analysis of variance tests were undertaken utilizing the general linear models procedure of the SAS® system for Windows 6.11.

Results

Lysozyme assay

The lysozyme activity in Atlantic salmon serum was found to have an optimum at pH 5.8 (see Fig. 1). Lysozyme activity of mucus was also pH dependent with an optimum at pH 6.2. HEWL in contrast, exhibited an optimal activity at pH 7.0 with sharper declines in activity at acidic and alkaline pH. While it is acknowledged, that because of the above results, HEWL may not be the best standard to use for lysozyme assays involving fish, it is readily available and consistently used by workers in the field. It was used in this study to maintain some comparability between results of the present studies and those reported in the literature.

A second order polynomial regression ($r^2 = 0.9862$) was fitted to the standard curve for HEWL to allow resultant optical density readings

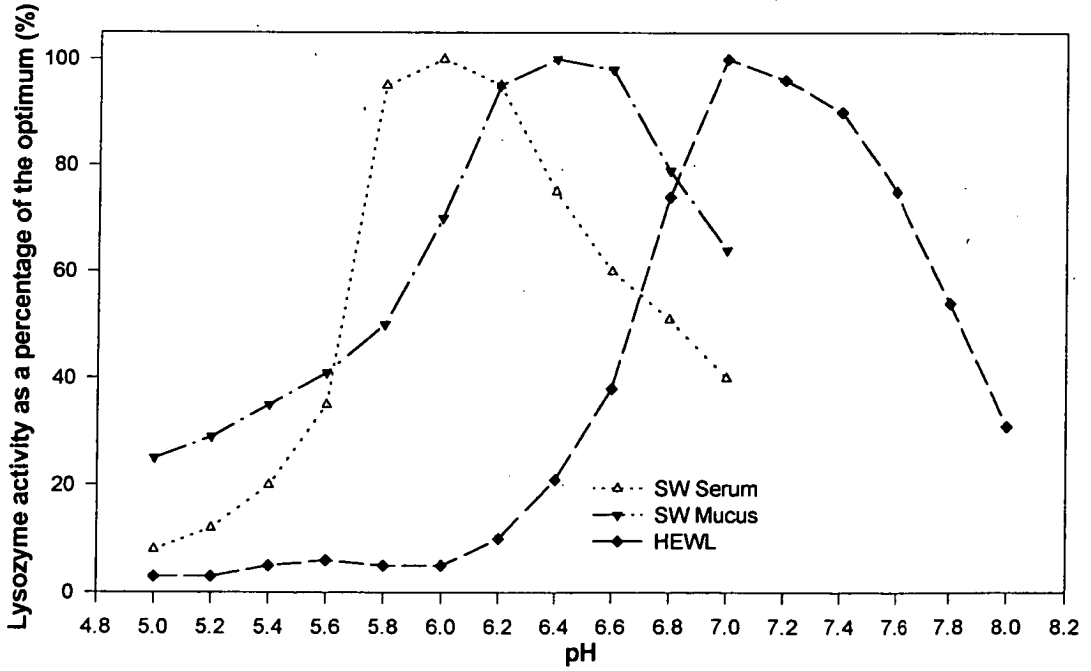

Figure 1 Effect of pH on the lysozyme activity of serum, mucus and HEWL.

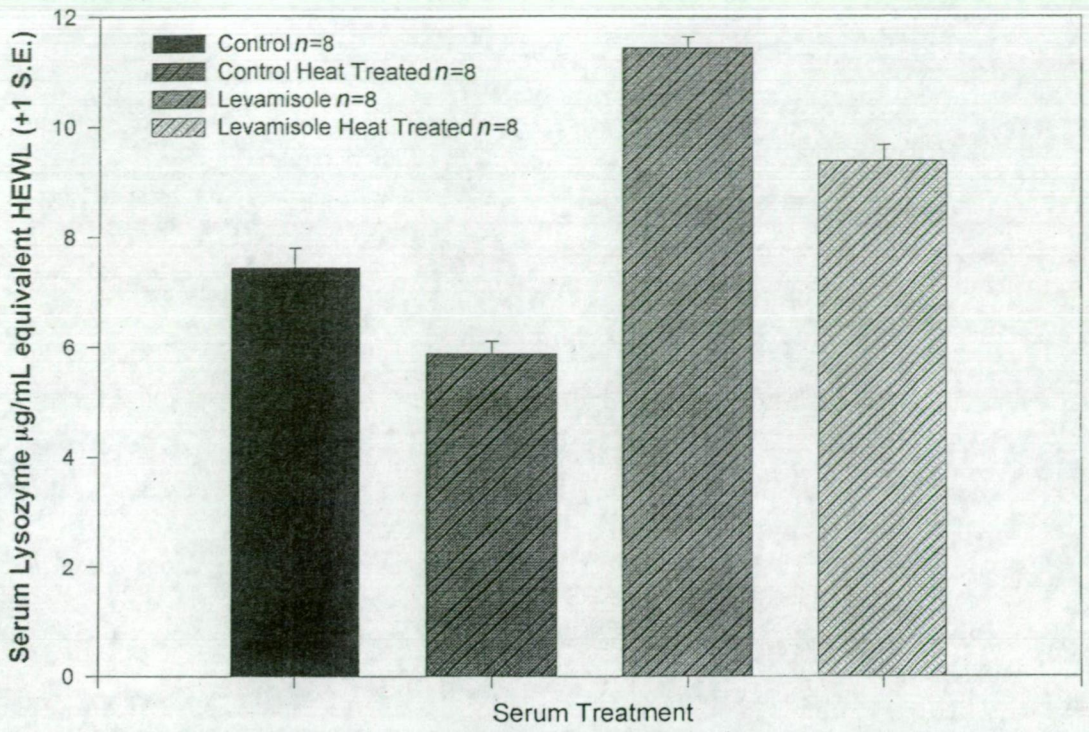

Figure 2 Lysozyme concentration of serum from Atlantic salmon treated with levamisole.

for serum and mucosal lysozyme to be converted to equivalent HEWL concentration. The following formula was used in this conversion: lysozyme concentration = 0.4185–5.5969 × change in OD + 65.3178 × change in OD^2. In all cases serum and mucus obtained from levamisole treated fish displayed a significantly increased lysozyme activity when compared to control fish ($F = 168.2$, d.f. = 1, $P < 0.0001$, $F = 425.44$, d.f. = 1, $P < 0.0001$ respectively) (see Fig. 2 and Fig. 3). Mucosal samples had consistently higher lysozyme activity than serum samples. Furthermore, when serum and mucus samples were heat-treated, thus inactivating the heat labile component, there was a consistent drop in the lytic activity whether they were levamisole treated or not ($F = 19.4$, d.f. = 2, $P < 0.0001$, $F = 34.13$, d.f. = 2, $P < 0.0001$ respectively) (see Fig. 2 and Fig. 3).

Phagocytosis assay

Head kidney cells that had been collected from the fish treated with levamisole demonstrated enhanced phagocytic abilities. Phagocytic ability was assessed and represented using measures of PI, PC and PA.

There was a significant increase in the number of yeast cells consumed per phagocyte in fish treated with levamisole ($F = 338.27$, d.f. = 1, $P < 0.0001$). The treated fish had a PI of 2.31 compared to control fish with a PI of 1.54. Levamisole treatment also significantly increased the phagocytic capacity of harvested phagocytes ($F = 375.02$, d.f. = 1, $P < 0.0001$). For phagocytes harvested from levamisole treated fish 84.75% had phagocytosed 1–3 yeast cells and 15.25% had phagocytosed 4–6+ yeast cells. For phagocytes harvested from control fish 97.05% had phagocytosed 1–3 yeast cells and 2.95% had phagocytosed 4–6+ yeast cells. Thus, for levamisole treated fish there was a right shift in the phagocytic profile of yeast consumption per phagocyte that represents an increased PC (see Fig. 4). Of the phagocytic cells harvested from fish that had been treated with levamisole, 50.03% had consumed one or more yeast cells. This is a significant increase ($F = 61.9415$, d.f. = 1, $P < 0.0001$) when compared to a phagocytic activity of 33.47% for control fish.

Superoxide anion production

In all experiments the addition of exogenous SOD inhibited the reduction of ferricytochrome C by the macrophages, confirming that the assay was specific for O_2^-. Figure 5 shows that the macrophages

from levamisole treated fish had a significantly increased O_2^- production with or without PMA triggering ($F = 209.66$, d.f. $= 3$, $P < 0.0001$). The Tukey's test distinguished between all treatments. Furthermore, at 24 h post-harvest the production of O_2^- in PMA stimulated macrophages from levamisole treated fish remained at elevated levels while all other groups returned to basal levels.

Haematocrit and leucocrit

No significant differences were found between levamisole treated and control fish for haematocrits and leucocrits ($F = 2.71$, d.f. $= 1$, $P = 0.1078$ and $F = 0.88$, d.f. $= 1$, $P = 0.3535$, respectively). Treated fish exhibited a mean haematocrit level of 48.8% and a mean leucocrit level of 1.78%. Control fish had a mean haematocrit level of 46.9% with a leucocrit value of 1.78%

Discussion

Levamisole has been shown to act as an immunostimulant in a number of fish species. Siwicki (1987, 1989) described the immunostimulatory activity of levamisole in carp spawners, with treated fish displaying elevated leucocyte and neutrophil numbers, enhancement of phagocytic activity, leucocyte migration and myeloperoxidase activity, increases of lysozyme levels and natural antibody titres. In their studies, Kajita, Sakai, Atsuta & Kobayashi (1990) showed that levamisole stimulated phagocytic activity, chemiluminescence responses and natural killer cell activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum). These workers demonstrated the activation of the alternative complement pathway. Levamisole also has an immunostimulatory effect on the sea bass, *Dicentrarchus labrax* L., and the gilthead seabream, *Sparus aurata* L., as shown by Jeney *et al.* (1994) and Mulero *et al.* (1998), respectively. Both groups demonstrated significant increases in respiratory burst and phagocytosis activities. The present study is the first record of the effect of levamisole on the nonspecific immune system of Atlantic salmon.

In this study all groups of fish given a bath treatment of levamisole had increased nonspecific defence factor values when compared to control fish. In those circumstances where the capacity to mount an immune response is not sufficient to successfully combat an invasion by pathogenic microorganisms, this demonstrated increase in the magnitude of the immune reaction may be adequate to ensure improved protection.

Granulocytes and mononuclear phagocytes or macrophages play a central role in the cellular part of the nonspecific defence of fish (Dalmo, Bog-

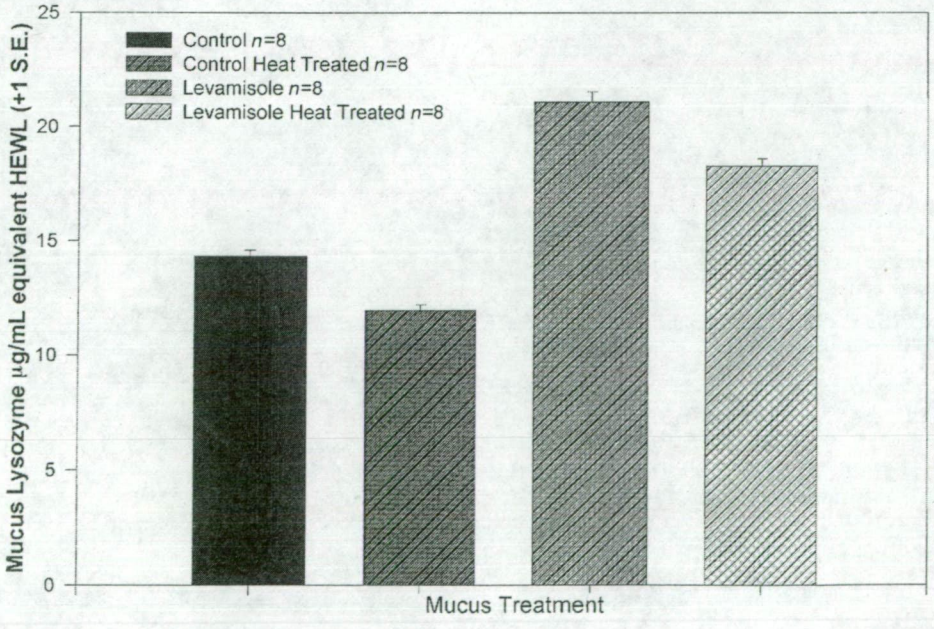

Figure 3 Lysozyme concentration of mucus from Atlantic salmon treated with levamisole.

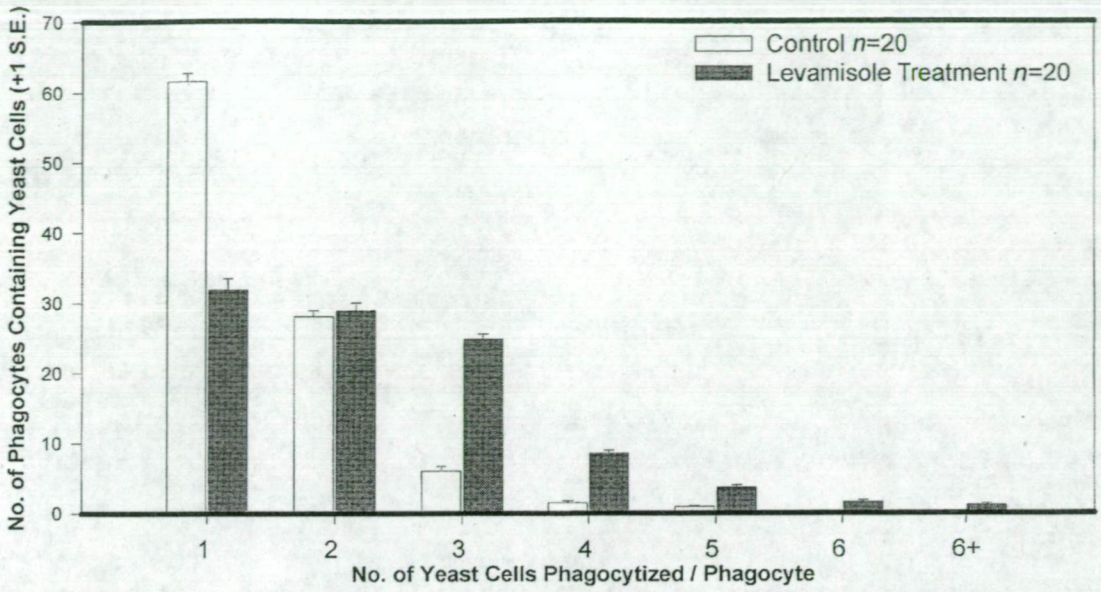

Figure 4 Activities of phagocytes from head kidneys of Atlantic salmon treated with levamisole.

wald, Ingebrigtsen & Seljelid 1996). In the present study, three phagocytic functions were assessed and, after treatment with levamisole, all were increased. Whilst phagocyte killing mechanisms are not well established in fish many studies have shown that fish phagocytes have potent bactericidal and larvicidal activity and thus, presumably, possess both intracellular and extracellular killing mechanisms (Secombes & Fletcher 1992). If extracellular killing can be facilitated then many implications may follow, particularly where parasites such as the causative agent of AGD infest areas that are relatively protected from the effects of antibody-mediated immunity. In this context, Whyte, Chappell & Secombes (1989) demonstrated that normal macrophages from rainbow trout are capable of killing non-opsonized diplostomes. Also, the release of free radicals such as superoxide anions and hydrogen peroxide and enzymes such as lysozyme may also play a role in extracellular killing. It may be relevant that Ellis (1982) suggested that the fish neutrophils may carry out a microbicidal role extracellularly rather than intracellularly.

If high levels of ROIs lead to the degeneration of parasitic invaders that would otherwise evade the immune system, then even if this killing were at the expense of cellular health, it must be seen as an advantage given the excellent regenerative powers of fish. Furthermore, Symoens & Rosenthal (1977) reported that levamisole treatment leads to an enhanced state of healing and wound repair.

While lysozyme is generally recognized as being part of the humoral nonspecific defence system it could be argued that it makes up part of the cellular nonspecific component given that it emanates from phagocytes. Lysozymes are widespread enzymes occurring in many teleost tissues and secretions (Lindsay 1986). With the exception of their probable antibacterial role, their function in vertebrates is still open to question (Jolles & Jolles 1984). Given the distribution of lysozyme in fish (i.e. in tissues rich in leucocytes and at sites where the risk of invasion is high such as skin, gills and gastrointestinal tract) it is logical to hypothesize that lysozyme provides a protective function especially as it has been demonstrated to be involved in defence against viruses, neoplasms, bacteria, fungi and insects (Dobson, Prager & Wilson 1984).

Prior to 1990, reports on the modulation of lysozyme activity in fish were limited (Mock & Peters 1990). Since that time, there has been an increase in the number of studies, perhaps reflecting the recognized importance of the nonspecific immune system in fish. This study confirms that levamisole can induce increased activities of both mucus and serum lysozyme. We will not attempt to compare the activity of lysozyme between fish species because there is such a great interspecies variation as shown by Grinde, Lie, Poppe & Salte

(1988), who reported the lysozyme levels of 12 fish with up to four fold variations between species. Furthermore, variation exists depending on the origin of the sample. Lindsay (1986) assayed lysozyme from the oesophagus, stomach, kidney, spleen, swim bladder and in the mucus and serum. The lowest levels reported were from serum and the highest were from the stomach. This pattern is compatible with a defence function and it seems likely that lysozyme is an integral part of the arsenal of nonspecific defence mechanisms of fish.

As part of the lysozyme assay, the heat labile component of both serum and mucus was also studied. Complement is an important part of this component and although the characterization of it is incomplete it is recognized as being relatively significant with respect to antimicrobial activity (Sakai 1992).

Complement acts as a membrane attack sequence that may be initiated by either the classical pathway that requires Ig to react with an antigen or via the alternative pathway with stimulation from a variety of substances. Heat treating mucus and serum samples prior to conducting the lysozyme assay did reduce the lytic activity but there was not a significant difference between levamisole treated and control fish, which suggests that levamisole had no effect on complement activity or other heat labile components.

Finally, the levels of haematocrit and leucocrit serve as a general indicator of fish health. These parameters are often used as confirmation that an immunostimulant is not disturbing the profile of the blood and thus homeostasis of the individual. No significant differences were found in the haematocrit or leucocrit levels between treated and control fish. Thus, it may be concluded that the modulatory effect of levamisole does not extend to a modification of the blood profile.

This study provides strong evidence that *in vivo* bath treatment with levamisole enhances the nonspecific immune system as measured by phagocytic ability, superoxide anion production and lytic lysozyme activity. While the present study has been conducted with the express interest of maximizing the effectiveness of AGD treatment (i.e. adding an immunostimulant to a therapeutic fresh water bath), it is acknowledged that, in cases where immunostimulation alone would be of sufficient benefit, oral treatment would be more practical. The findings in this study are in accordance with the previously discussed studies of the effects of levamisole in other fish species. The results of this study may prove to be of practical value given the

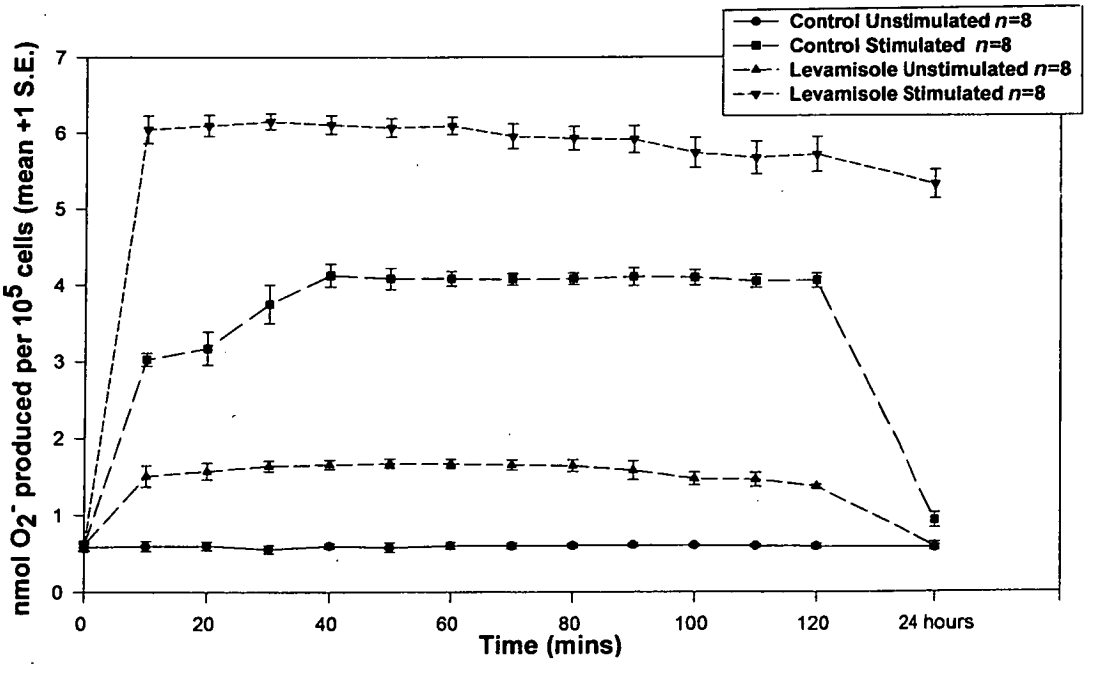

Figure 5 Superoxide anion production by Atlantic salmon head kidney macrophages.

efficacy of this drug at such low concentrations and the ability to treat large quantities of fish at any given time. Furthermore, the prophylactic use of levamisole may be of value where situations known to result in stress and exposure to disease occur.

Acknowledgements

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Effects of Treatment with Levamisole and Glucans on Mortality Rates in Atlantic Salmon (*Salmo salar* L.) Suffering from Amoebic Gill Disease.

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Abstract

When levamisole was added to freshwater baths, used to treat Atlantic salmon for amoebic gill disease (AGD), there was a significant decrease in subsequent mortality of the fish. This was particularly the case in fish experiencing their first exposure to AGD. There were no significant differences in responses to 1.25, 2.5 and 5.0 mg/L levamisole. It is proposed that this response is related to enhancement of the non-specific immune system. Oral supplementation with levamisole or glucans did not prevent subsequent mortalities when Atlantic salmon, naïve to AGD were subsequently exposed to this disease.

Introduction

Amoebic gill disease (AGD) is the most important disease of sea-caged Atlantic salmon in Tasmania (Findlay and Munday 1998) and is currently treated and controlled by the use of freshwater bathes and/or brackish water culture sites. There have been a number of attempts to find suitable chemotherapeutants which can be fed to the fish or used in seawater (Alexander 1991, Cameron 1994, Howard and Carson 1994), but the only encouraging results have been those of Lee *et al* (1994) and Lee (1995) who researched oral supplementation with ß-glucans, which are known to be stimulators of the non-specific immune system in fish (Robertson *et al.* 1990, Nikl *et al.* 1991, Raa *et al.* 1996). This paper reports studies using ß-glucans and levamisole, another compound which acts as an immunostimulant of the non-specific immune system in fish (Siwicki 1987 and 1989, Kajita *et al.* 1990, Jeney *et al.* 1994, Mulero 1998, Findlay and Munday 2000), as potential prophylactic treatments for AGD. The information on mortalities reported here was gathered incidentally to a major series of trials to be reported elsewhere, in which the measurement of disease was the extent of gill lesions.

Materials and Methods

Atlantic salmon of 100-200 g bouy weight (BW) and naïve in relation to AGD were used in all the trials. The major part of the study was based on the experimental design de-

scribed by Findlay and Munday (1998). In brief, fish which had previously experienced no, one or two episodes of AGD were given a variety of freshwater bath treatments with different levels of levamisole, and then re-exposed to infection in the form of cohabitation with clinically diseased fish. Details of the three individual trials are given in Table 1. Mortalities were recorded over a four-week period. In a completely separate trial, fish were divided into three groups, each held in freshwater in 4000 Rathburn recirculating tanks. All fish were fed with commercial salmon feed, at the rate of 1% BW, supplemented as follows: 1. ß- glucans at 1g/kg feed, commencing 31 days before exposure to AGD for a period of 21 days (i.e. no supplementation for the last 10 days). 2. Levamisole at 500 mg/kg feed commencing 20 days before exposure to AGD for a period of 10 days (i.e. no supplementation for the last 10 days). 3. Unsupplemented salmon feed. The three groups were split between two tanks, acclimatized to seawater, and cohabited with fish with clinical AGD. Mortalities due to AGD were recorded over a three-week period. Details of the group sizes used are given in Table 2. Mortality patterns were similar between replicate tanks in individual trials, therefore the data for each treatment within an experiment were combined. Treatments were compared by the chi-squared test.

Results

In the trials within the major integrated project (Table 1), it was found that both the use of levamisole in freshwater bath, and previous history of the fish in relation to AGD influenced the results (Table 1). In all instances, when naïve fish (which had never previously experienced AGD) bathed in freshwater alone, were cohabited with fish with clinical AGD, they suffered significantly greater mortalities in comparison to all other groups, including naïve fish which were bathed in freshwater containing 5.0 mg/L levamisole (Table 1, trials 1 and 2). Because of the varying severity of disease induced in the different trials (1, 2 and 3), the other results were not so clear cut, but the main findings were: 1. Within the range of 1.25-5.0 mg/L, the concentration of levamisole in the freshwater bath did not appear to affect the outcome (Table 1, trial 3). 2. The addition of levamisole to a freshwater bath appeared to reduce mortalities if the fish were naïve to AGD or had previously suffered one episode of AGD. (Table 1, trials 2, 3 and trial 1 with naïve fish groups, but not previously exposed groups). Levamisole had no significant effect if the fish had experienced two episodes of AGD or had experienced one episode and had been returned to freshwater for four weeks before re-exposure to AGD (table 1, trial 2). In contrast to the apparent efficacy of levamisole in freshwater baths in reducing mortalities, this chemotherapeutant and glucans given orally did not appear to provide useful protection against mortalities due to AGD (Table 2).

Discussion

As reported previously by Findlay and Munday (1998), the resistance developed by salmonids to reinfection with AGD appears to be due to stimulation of the fish's non-specific immune system. As both levamisole (Siwicki 1987 and 1989, Kajita *et al.* 1990, Jeney *et al.* 1994, Mulèro 1998, Findlay and Munday 2000) and glucans (Robertson *et al.* 1990, Nikl

Trial no.	Treatment	Levamisole dose (mg/l)	Total number of fish (two replicates)	Total mortalities	RPP%
1	PE x 1 Lev	5.00	36	1[b]	97.2
1	PE x 1 FW	0	36	1[b]	97.2
1	Naï ve Lev	5.00	36	2[b]	94.4
1	Naï ve Lev	0	36	8[a]	77.8
2	PE 4FW Lev	5.00	30	0[c]	100
2	PE 4FW FW	0	30	0[c]	100
2	PE x 2 Lev	5.00	30	0[c]	100
2	PE x 2 FW	0	30	0[c]	100
2	PE x 1 Lev	5.00	30	2[c]	93.3
2	PE x 1 FW	0	30	30[a]	0
2	Naï ve Lev	5.00	30	7[b]	76.6
2	Naï ve FW	0	30	30[a]	0
3	PE Lev	5.00	36	0[c]	100
3	PE Lev	2.50	31	0[c]	100
3	PE Lev	1.25	33	0[c]	100
3	PE FW	0	34	6[b]	82.4
3	Naï ve FW	0	40	15[a]	62.5

Table 1: Summary data of experimental procedures and mortalities for levamisole supplementation in freshwater bath treatments. [abc] different superscript indicates a significant difference within a trial. PE= previously exposed, FW= 2-3h freshwater bath only, Lev= 2-3h freshwater bath + levamisole, 4FW= 4 weeks in freshwater before re-exposure, RPP= relative percent protection.

et al. 1991, Raa *et al.* 1996) are capable of stimulating the non-specific immune system in their own right, it would be logical that they would be useful in the treatment and prevention of AGD. However it appears that oral administration of these immunomodulators is not as efficiouse as the use of levamisole in a freshwater bath, where it, presumably, acts locally on the gills. Unfortunately, the authors were unable to obtain soluble glucans preparations for use in baths, but an attempt to use standard preparations suggested that glucans may also be efficient by this route (Findlay unpublished). While these results are encouraging, their application in the field is not straightforward because farms may not know how many episodes of AGD their fish have been previously exposed to. The possibility of using levamisole during transport of smolts from the hatchery to their sea-cages is worthy of consideration. In that instance, if low salinity water is used as the transport medium, it would be necessary to ensure that the water was not sufficiently alkaline to hydrolyze the levamisole.

Treatment	Total number of fish (two replicates)	Total mortalities	RPP%
Glucans	30	11[a]	63.3
Levamisole	33	16[a]	51.5
Control	30	14[a]	53.3

Table 2: Summary data of experimental procedures and mortalities for glucans and levamisole supplementation in feed. [abc] different superscript indicates a significant difference. RPP= relative percent protection.

Acknowledgements

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Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*

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Abstract

Amoebic gill disease (AGD) of maricultured salmonids, turbot, *Scophthalmus maximus* (L.), European seabass, *Dicentrarchus labrax* (L.), and sharpsnout seabream, *Diplodus puntazzo* (Cetti), caused by *Neoparamoeba pemaquidensis* has been reported from Australia (Tasmania), Ireland, France, Chile, North America (Washington State and California) and Spain. Of the salmonids, Atlantic salmon, *Salmo salar* L., appears to be the most susceptible with rainbow trout, *Oncorhynchus mykiss* (Walbaum), also suffering significant disease. Only minor outbreaks have been reported in coho, *O. kisutch* (Walbaum), and chinook salmon, *O. tshawytscha* (Walbaum). The disease now accounts for 10–20% of production costs of Atlantic salmon in Tasmania and has lead to temporary abandonment of culture of this species in parts of Spain. It is of lesser, but still significant, importance in other countries. Much is known about the pathology of AGD but the pathophysiology of the disease is poorly understood. There is evidence that non-specific immunity is involved in fish acquiring resistance to AGD, but no unequivocal evidence exists for protection as a result of specific immune responses. To date, for salmonids, the only effective treatment for AGD is a freshwater bath. Control procedures based on modification of management strategies have been minimal and virtually unresearched.

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Historical background

Amoebic gill disease (AGD) of sea-caged Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), was first described by Munday (1986) soon after Atlantic salmon culture was initiated in Tasmania. The causative amoeba was not further identified at that time but subsequently Roubal, Lester & Foster (1989) ascribed it to the genus *Paramoeba*. In the intervening period Kent, Sawyer & Hedrick (1988) described AGD in coho salmon, *O. kisutch* (Walbaum), in Washington State and California and identified the aetiological agent as *P. pemaquidensis*, although Page (1987) had redescribed the organism as *Neoparamoeba pemaquidensis*. The development of immunohistochemical techniques by Howard & Carson (1993) simplified identification of *N. pemaquidensis* and the organism was subsequently identified as the cause of AGD in Atlantic salmon in Ireland and Chile and in chinook salmon, *O. tshawytscha* (Walbaum), in New Zealand by use of the indirect fluorescent antibody test (IFAT).

Initially, the disease caused devastating losses in Tasmanian sea-caged salmonids but the introduction of freshwater bathing reduced losses considerably, although the financial impact was still great (Munday, Foster, Roubal & Lester 1990). However, recent increases in stocking densities together with sequential introductions of naïve fish (spring smolts, out-of-season smolts and pre-smolts) and warm summers have coincided with

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worrying losses and costs which now amount to 10–20% of production costs. Similarly, in the warm Spanish waters AGD has made Atlantic salmon farming potentially unviable (Arán, personal communication).

Amoebic gill disease of turbot, *Scophthalmus maximus* (L.), was first reported by Dykova, Figueras & Navoa (1995), but it was not until 1998 that the causative agent was identified as a *Paramoeba* sp. (Dykova, Figueras, Novoa & Casal 1998). The disease has also been reported in European seabass, *Dicentrarchus labrax* (L.), and sharpsnout seabream, *Diplodus puntazzo* (Cetti), from the Mediterranean (Dykova, Figueras & Peric 2000; Dykova & Novoa 2001).

Species affected and geographical distribution of AGD

Atlantic salmon is apparently the salmonid species most susceptible to AGD. Outbreaks of disease in this species have been reported from Tasmania (Munday, Lange, Foster, Lester & Handlinger 1993), Ireland (Rodger & McArdle 1996; Palmer, Carson, Ruttledge, Drinan & Wagner 1997), France (Findlay & Munday 1998), Chile (Nowak 2001a), Spain (Rodger & McArdle 1996; Arán, personal communication) and Washington State, USA (Douglas-Helders, Saksida, Raverty & Nowak 2001). The disease has not been reported from Canada, Iceland, Scotland or Norway, probably because the water temperatures are generally lower at these locations. In some instances other salmonid species cultured alongside Atlantic salmon in the areas where AGD is present have not had clinical AGD or it has been considered of minor importance (F. Baudin-Laurencin, personal communication).

Rainbow trout in Tasmania suffered severe disease because of *N. pemaquidensis* infection and that, together with high water temperatures, has limited the production of 'ocean trout' in Tasmania. In France high water temperatures have been the more frequent limiting factor with this species (Baudin-Laurencin, Aldrin, Messager & Tuffrant 1985) although AGD has occurred sporadically (Baudin-Laurencin, personal communication).

Sea-caged brown trout, *S. trutta* L., are only cultured to a significant extent in France and AGD has been diagnosed in that species (Baudin-Laurencin, personal communication).

Apart from outbreaks in rainbow trout very little AGD has been reported from *Oncorhynchus* spp. Despite the fact that New Zealand produces a significant tonnage of salmonid product under very similar environmental conditions to those existing in Tasmania, only a few minor outbreaks of AGD have been diagnosed in the chinook salmon cultured in that part of Australasia. Only one significant outbreak of disease was described by Kent *et al.* (1988) in the coho salmon monitored in Washington State and California from 1985 to 1987.

Amoebic gill disease caused by *N. pemaquidensis* has occurred as a problem of turbot culture in north-west Spain since 1995 (Dykova *et al.* 1995, 1998). No such problem has been reported with turbot culture in France, although the disease is recognized in salmonids (Baudin-Laurencin, personal communication). Only fleeting reference has been made to AGD in European seabass and sharpsnout seabream (Dykova & Novoa 2001).

The only report of amoebae consistent with *Neoparamoeba* on wild fish was that of Foster & Percival (1988b) who observed the organisms on the gills of immature couta, *Thyrsites atun* (Euphrasen), in the vicinity of infected salmonids in Tasmania. Red cod, *Pseudophycis bachus* (Forster), jack mackerel, *Trachurus declivis* (Jenyns), Tasmanian blenny, *Picticlennius tasmanianus* (Richardson), and sand flathead, *Platycephalus bassensis* (Cuvier), caught in the vicinity of, and remote from, sea cages have not been found to have amoebae on their gills (Dawson 1999; Nowak 2001b; Munday & Woodworth, unpublished observations). However, it has proven possible to experimentally produce gill infection, but not AGD, with *N. pemaquidensis* in greenback flounder, *Rhombosolea tapirina* (Günther), and big-bellied seahorses, *Hippocampus abdominalis* (Lesson) (Nowak, Douglas-Helders & Dawson 2000).

Clinical and pathological features

Peak mortalities in Atlantic salmon smolts in Tasmania have been 10% per week with losses of 2–4% per week in fish weighing 1–2 kg and 1–2% per week in fish over 2 kg (Foster & Percival 1988b).

Clinical signs of lethargy and respiratory distress manifested as rising to the surface of the water and increased rate of opercular movement have been described in salmonids by Kent *et al.* (1988),

Figure 1 Light amoebic gill disease infection in an Atlantic salmon. Arrows point to small, discrete patches typical of the disease.

Figure 2 Heavy amoebic gill disease infection in an Atlantic salmon. Some patches have coalesced.

Figure 3 Photomicrograph of amoebic gill disease showing lamellar fusion and the presence of amoebae which are only identifiable by their shape (H&E, bar = 100 μm).

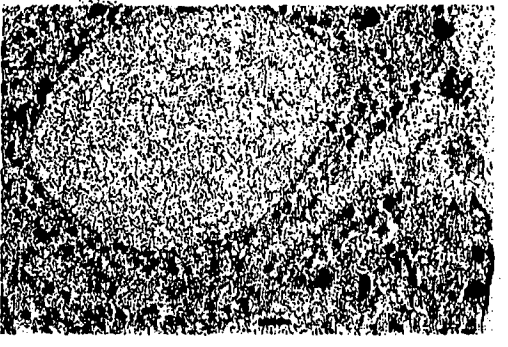

Figure 4 Photomicrograph of amoebic gill disease showing a cyst formed as a result of lamellar fusion. Note staining of amoebae which makes them more readily identifiable (PAS/Alcian blue, bar = 25 μm).

Munday *et al.* (1990) and Rodger & McArdle (1996). However, in experimental studies Powell, Fisk & Nowak (2000) were unable to confirm that AGD-infected fish actually had a measurable increase in ventilation frequency. In Atlantic salmon macroscopic lesions are usually multifocal patches of white to grey, swollen gill tissue with associated excess mucus (Figs 1 & 2) (Munday *et al.* 1990; Rodger & McArdle 1996). These are most numerous in the dorsal portions of the gill arch (Adams 2000; Adams & Nowak 2001). In rainbow trout the mucoid branchitis is more diffuse. Histopathological descriptions of the disease in salmonids and turbot have been remarkably similar with the main lesion being hyperplasia and hypertrophy of the gill epithelium (Fig. 3) (Kent *et al.* 1988; Munday *et al.* 1990; Dykova *et al.* 1995; Rodger & McArdle 1996; Zilberg & Munday 2000). Lamellar fusion is also prominent often leading to the formation of cystic spaces (Fig. 4). In particular, the description of the experimental disease by Zilberg & Munday (2000) provides information on the sequential development of lesions and will be paraphrased here. At 2 days after exposure to AGD-infected fish *Neoparamoeba* could be seen on the gills, which were otherwise normal. By 4 days post-exposure (DPE) multifocal hyperplasia and lamellar fusion was present and this involved up to 15 gill lamellae per focus by 7 DPE. At that time the epithelial cells were both hyperplastic and hypertrophic and the tissues were spongiotic. *Neoparamoeba* became more abundant, associated mainly with the hyperplastic epithelium and, in some instances, were sloughed off with hyperplastic tissue. This 'self-cleansing' action may be important in both natural and iatrogenic recovery from the disease. Also, a marked increase in mucous cells was noted. At 28 DPE epithelial hyperplasia and lamellar fusion was extensive with numerous associated *Neoparamoeba* sp. (Fig. 5).

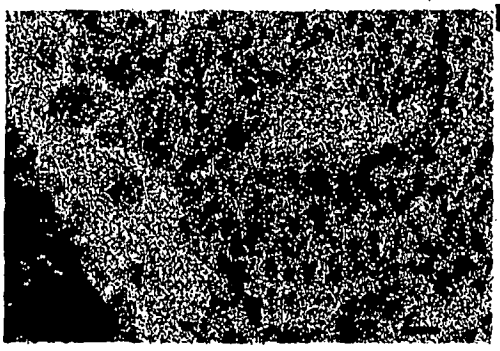

Figure 5 Photomicrograph of amoebic gill disease showing amoebae staining the same as the gill tissues. (H&E, bar = 25 μm).

Other authors noted the development of interlamellar vesicles often containing amoebae and inflammatory infiltrates in the supporting tissues (Munday *et al.* 1990; Adams & Nowak 2001). Severely affected fish have been shown to have longer ventricles in comparison with their ventricular width or height compared with minimally affected fish (Powell & Nowak 2001). This macroscopic change is accompanied by thickening of the compact ventricular muscle.

The crucial metabolic perturbations associated with AGD are still unclear. Munday *et al.* (1990) reported elevated blood sodium levels in severely affected fish and Powell *et al.* (2000) found a respiratory acidosis, but in both instances the abnormalities were not widespread and severe enough to explain the clinical signs.

More recently Powell & Nowak (2001) have found that affected fish are hypertensive and this condition is ameliorated by freshwater bathing. This effect on the vascular system is perhaps comparable with the situation in bacterial gill disease where exotoxins produce vascular constriction (Byrne, Ostland, Lumsden, MacPhee & Ferguson 1995).

The aetiological agent

Kent *et al.* (1988) and Foster & Percival (1988b) reported that the causative agent was a *Paramoeba* sp., probably *P. pemaquidensis*. Subsequently, Howard & Carson developed a polyclonal antibody against a Tasmanian isolate of the agent and this reacted with the agents present in Ireland (Rodger & McArdle 1996), New Zealand and France (T. Howard & J. Carson, personal communication).

More recently, Wong & Elliot 2000) and Elliott, Wong & Carson (2001) have developed a PCR which identifies *N. pemaquidensis* with great specificity. This assay was developed after the complete 18S rDNA gene sequence was obtained for the type *N. pemaquidensis* and identifies the organisms recovered from AGD of salmonids in Australia, Ireland and Washington State and turbot in Spain as being identical. The salmonid isolates were shown to share 98–99% sequence similarity over 2104 base pairs of the 18S rDNA gene (Elliott *et al.* 2001). However, as the American and Spanish organisms can survive in much lower salinities than the Australian *N. pemaquidensis* it appears that the PCR may not distinguish between biovars with different physiological characteristics. Also, Dykova *et al.* (2000) were unable to distinguish six strains of *N. pemaquidensis* from fish with AGD from the type strain of *N. aestuarina* by electron microscopy.

Amoebae freshly removed from infected gills appear as subspherical (15–40 μm diameter), transitional forms with up to 50 digitate pseudopodia (Kent *et al.* 1988; Munday *et al.* 1990; Rodger & McArdle 1996; Dykova *et al.* 1998) (Fig. 6). The organisms possess a nucleus (≈ 5 μm diameter) and one or more parasomes (≈ 4 μm). The latter have been identified as the symbiont *Perkinsella amoebae* (Dykova *et al.* 2000).

Both Munday *et al.* (1990) and Dykova *et al.* (1995) described the amoebae in histological sections as being vacuolated and frequently closely adherent to the gill epithelium, an observation confirmed by electron microscopy (Roubal *et al.* 1989).

These gill-associated *N. pemaquidensis* have been grown on lawns of numerous bacterial species on

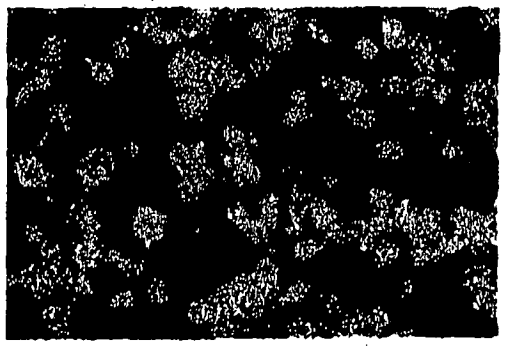

Figure 6 Photomicrograph of granular, live amoebae admixed with erythrocytes. The presence of pseudopodia on the amoebae is diagnostic (unstained, bar = 30 μm).

malt–yeast–sea water agar by a number of workers (Kent *et al.* 1988; Munday *et al.* 1990; Dykova *et al.* 1998). Attempts to produce AGD with cultured organisms have been uniformly unsuccessful (Kent *et al.* 1988; Howard, Carson & Lewis 1993; Findlay 2001) and this has only be achieved by cohabiting naïve fish with infected fish (Howard *et al.* 1993; Akhlaghi, Munday, Rough & Whittington 1996; Findlay 2001) or by exposing them to isolated, gill-associated *N. pemaquidensis* (Zilberg, Gross & Munday 2001).

Very few detailed studies have been undertaken on the organisms to identify possible virulence factors. The presence of extracellular products is assumed but has not been demonstrated. A galactose-specific lectin has been found in cultured *N. pemaquidensis* (Cane, personal communication) and could be involved in expression of pathogenicity. Also, Dykova *et al.* (2000) noted that the number of *N. pemaquidensis* with fully developed *P. amoebae* decreased as the cultures aged suggesting that the metabolic state of the *Neoparamoeba* had changed.

Diagnosis

In the first instance it is important to distinguish between AGD and the presence of *N. pemaquidensis* with no or inconsequential gill pathology. For the aquaculturist the former is the practical diagnosis whereas clinicians and, particularly, researchers have an interest in both these aspects of diagnosis. In some instances diagnosis of AGD is relatively simple but at other times, as with most diseases, differential diagnosis depends upon a proper and balanced consideration of all factors and observations.

On-farm diagnosis in Atlantic salmon is usually by counting the typical mucoid patches on the gills (Alexander 1991) (Fig. 1). While this has been shown to be highly correlated with the presence of *N. pemaquidensis* in experimental infections (Zilberg & Munday 2000; Findlay 2001), the association is inconsistent in the field (Clark & Nowak 1999). Also, this technique is not applicable to rainbow trout (Munday *et al.* 1990) or turbot (Dykova & Novoa 2001).

The presence of presumptive *N. pemaquidensis* can be confirmed by examining wet mounts taken from fresh gills (Fig. 6), staining with Quick Dip® (Fronine Pty Ltd) (Zilberg, Nowak, Carson & Wagner 1999) or histology. The former suffers from the problem that amoebae can be difficult to differentiate from gill epithelial cells and typical amoeboid shapes are rarely seen in smears from fish which have been dead for some time (Munday *et al.* 1993). In experimental infections, histology reveals amoebae attached to the gill epithelium at 2 days after exposure and this is the earliest any method confirms the infection (Zilberg & Munday 2000). Histologically, the presence of amoebae can be better appreciated by staining with PAS/Alcian blue (Figs 4 & 5). It is not unusual for other protozoa, including other genera of amoebae, to be present as mixed infections with *N. pemaquidensis* (Munday *et al.* 1993; Dykova & Novoa 2001).

The IFAT and dot-blot techniques have been developed for diagnosis of *N. pemaquidensis* infections using polyclonal antisera against the pathogen. However, the IFAT does not give a positive result until 7 DPI (Zilberg & Munday 2000) and the dot-blot cross-reacts with *N. destructa* and *Pseudoparamoeba pagei* (Douglas-Helders, Carson, Nowak & Wagner 2000; Douglas-Helders, Carson, Howard & Nowak 2001). A specific PCR has been developed (Wong & Elliott 2000; Elliott *et al.* 2001) and is suitable for use with fish gills, sea water and biofouling.

Pathogenesis

It is apparent from the studies of Zilberg & Munday (2000) that *N. pemaquidensis* is capable of colonizing the normal gill epithelium, probably by virtue of a lectin/glycoconjugate bond. It is likely that the glycoconjugate is galactose or *N*-acetyl-galactosamine (Findlay 2001). As Padilla-Vaca, Ankri, Bracha, Koole & Mirelman (1999) have shown that the glycoconjugate profile of the bacteria used as the nutritional lawn for cultured *Entamoeba histolytica* is crucial in deciding if the organism is to be pathogenic or not, similar studies are warranted for *N. pemaquidensis*. This mechanism may also be involved in the apparent preference of *N. pemaquidensis* for hyperplastic gill epithelium (Munday *et al.* 1990; Nowak & Munday 1994; Dykova *et al.* 1995). Indeed, a vicious cycle seems to develop whereby the infection leads to the production of hyperplastic gill epithelium which attracts more amoebae and so on until the fish dies, immunity intervenes and/or the environmental conditions become less suitable for the pathogen. This will be discussed further under 'Epidemiology'.

Epidemiology

The epidemiology of AGD will be discussed according to parasite, host and environmental variables.

The parasite

There is some evidence from experimental studies that *N. pemaquidensis* becomes more virulent with sequential passage through naïve hosts (Findlay, Zilberg & Munday 2000). This may have profound implications for current husbandry in Tasmania where pre-smolts (conditioned in brackish water), spring smolts and out-of-season smolts are sequentially introduced onto farms.

The main source of infective organisms is undoubtedly infected fish. Zilberg *et al.* (2001) have shown that the minimum infectious dose for Atlantic salmon is about 230 *N. pemaquidensis* L^{-1}. When it is realized that a single smolt with AGD may be carrying between several hundred thousand to a few million amoebae, the danger posed by a cage of infected fish can be appreciated. Even dead fish carry live *N. pemaquidensis* for up to 3 days postmortem (Douglas-Helders, Nowak, Zilberg & Carson 2000; Dykova & Novoa 2001), so the danger does not cease with the demise of the fish.

Foster & Percival (1988b) reported that clean nets but not fouled nets supported colonies of amoebae and this has essentially been confirmed by Tan, Nowak & Hodson (2000; personal communication) who found that even nets treated with copper-based antifoulants supported more *Neoparamoeba* than untreated nets. Thus, fouled nets do not appear to constitute a significant reservoir of infection.

There is some evidence that more severe AGD is associated with high algal counts or chlorophyll levels in the water column (Nowak 2001b; S. Percival, personal communication).

The host

As mentioned under 'Species affected and geographical distribution of AGD' Atlantic salmon and rainbow trout appear to be the most susceptible salmonids. There is good field and experimental evidence that previously exposed fish acquire a degree of resistance to re-infection after freshwater bathing (Foster & Percival 1988a; Findlay, Helders, Munday & Gurney 1995; Findlay & Munday 1998). However, this is only relative and can be overcome by excessive challenge. Sexually mature fish appear to be more susceptible, whereas so-called 'pinheads' have fewer lesions than normal fish in the same cages (Mitchell 2001; Percival, personal communication).

Minor hyperplastic lesions on the gills are associated with some increase in severity of AGD (Nowak & Munday 1994; Zilberg & Munday 2000) but are probably not significant in deciding the eventual outcome. However, severe gill lesions resulting from jellyfish damage and clubbing and necrotic gill syndrome are sometimes rapidly colonized by *N. pemaquidensis* with drastic consequences (J. Handlinger, personal communication).

Environment

The most consistent association for clinical AGD is with water temperature (coupled with the appropriate salinity). In general, outbreaks in salmonids have only occurred at water temperatures of 12–20 °C (Kent *et al.* 1988; Munday *et al.* 1990; Rodger & McArdle 1996), although amoebae can be found on the gills at 10 °C (Munday *et al.* 1990) and Clark & Nowak (1999) and Douglas-Helders *et al.* (2001) reported AGD in Atlantic salmon at water temperatures in the range 9.1–10.6 °C. In experimental infections the disease can be effectively manipulated within a range of 12–14 °C (Findlay 2001) but at 16–18 °C it would be very difficult to control (Aldhlaghi *et al.* 1996; Munday, unpublished observations). The AGD was recorded in turbot at maximum temperatures ranging from 14 to 18.8 °C (Dykova *et al.* 1998).

The situation in relation to salinity is intriguing. All reported long-term infections in salmonids have been associated with high salinity (≥ 32‰) sea water (Clark & Nowak 1999; Foster & Percival 1988b; Munday *et al.* 1990; Rodger & McArdle 1996; Zilberg & Munday, unpublished observations). In contrast, AGD in turbot has occurred at a constant salinity of 22‰, a salinity at which amoebae disappear from the gills of infected rainbow trout within 24 h (Munday & Hortle, unpublished observations).

Treatment

Foster & Percival (1988a) were the first to document the efficacy of freshwater baths in the treatment of salmonid AGD. They recommended a

bath of 2–6 h duration with close to zero salinity water. They also emphasized the need for gentle handling of the fish, the need to maintain high levels of dissolved oxygen and the hazards of water with high iron content or gas supersaturation. Cameron (1993) further qualified the data relating to salinity and suggested that salinities greater than 4‰ were associated with unsatisfactory control of AGD. More recently, Powell (personal communication) has found that *N. pemaquidensis* survives better in water rich in $CaCl_2$ and/or $MgCl_2$. Munday *et al.* (1990) suggested that freshwater baths had three effects:

1. To reduce the number of amoebae on the gills.
2. To remove sea water-stable mucus from the gills.
3. To reduce any hypernatraemia which may have developed.

Although Howard & Carson (1993) found that *N. pemaquidensis* was inactivated by exposure to reagent grade freshwater, more recent studies have shown that up to 27% of the amoebae remain viable after normal on-farm freshwater baths and these organisms are capable of initiating recurrent AGD under experimental conditions (Clark, Nowak & Powell 2000; Findlay 2001; Parsons, Powell, Fisk & Nowak 2001). Thus, killing of the amoebae on the gills seems to be of lesser importance in comparison with returning the gill epithelium to normality as a result of treatment.

Removal of mucus from the gills seems to be an important function of a freshwater bath. This process removes amoebae mechanically in a manner similar to natural exfoliation of gill mucus (Zilberg & Munday 2000) and in the short term it potentially alleviates the physiological perturbations produced by AGD (Powell *et al.* 2000; Powell & Nowak 2001). It is probable, but unproven, that the replacement mucus is rich in such immune components as lysozyme which help inhibit recolonization of the gills by *N. pemaquidensis*.

As only severely affected fish have hypernatraemia (Findlay 2001), reversal of this change is likely to be of only minor importance.

A large number of treatments other than freshwater have been trialled *in vivo* and *in vitro*, but as there has not been universal correlation between these only the *in vivo* studies will be considered here. Table 1 provides documentation on the treatments which have been tried and found to be ineffective in relieving clinical AGD at the concentrations/dose rates used. The only compounds found to date to influence pre-existing AGD have been narasin [(4S)-4-methylsalinomycin)] and levamisole [1–2,3,5,6-tetrahydro-6-phenyimadazo (2,1-*b*) thiazole].

Cameron (1992) reported that narasin fed at the rate of 50–60 mg kg^{-1} bodyweight for 7 days reduced AGD gill lesions but there were palatability problems and the trials were not persisted with. Cameron (1992) also trialled oral levamisole (15 mg kg^{-1} bodyweight every third day for 15 days) without benefit for infected fish. In contrast, Findlay *et al.* (2000) found that 1.25–5 p.p.m. levamisole in freshwater for 2–3 h significantly augmented the efficacy of the freshwater bath, especially in fish that had been in the sea for a short period and had only limited exposure to *Neoparamoeba*. Based on studies by Akhlaghi *et al.* (1996), Findlay & Munday (2000) and Zilberg & Munday (unpublished observations) it is presumed that levamisole acts by stimulating the non-specific immune system. However, Clark

Table 1 *In vivo* treatments found to be ineffective against amoebic gill disease

Type of treatment	Specific agent used for treatment
Antimicrobials	Albendazole (o), amprolium (o), fumagillin (o), juglone (o), malachite green (b), mebendazole (b), metronidazole (o), quinacrine (b, o), Rometrimethoprim (o), toltrazuril (b), triclabendazole (o), 8-hydroxyquinolone (o)
Detergents	Alkadet 15 (b), LWA 1570 (b), Tween 20 (b)
Disinfectants	Copper sulphate (b), chelated copper (b), chloramine T (b), formalin (b), hydrogen peroxide (b), potassium permanganate (b)
Mucolytic	L-cysteine ethyl ester (o)

b = Bath (added to sea water), o = oral. Sources of information: Alexander (1991), Cameron (1992, 1993), Foster & Percival (1988a), Munday *et al.* (1993), Zilberg & Munday (unpublished observations).

& Nowak (1999) were unable to detect a beneficial effect of levamisole under field conditions which emphasizes the strict criteria which must be met for this immunomodulator to exert a beneficial effect. Preliminary trials with higher doses of levamisole in sea water adjusted to pH 7 with citric acid have yielded promising results by returning the gill epithelium to normal, although some toxicity has been noted (Munday & Zilberg, unpublished observations). It is also possible that the increase in gill mucous cell numbers reported by Morrison, Nowak & Carson (2001) may play a role in the therapeutic effect of levamisole.

Chlorine dioxide and chloramine T added to freshwater baths increase the amoebacidal capacity of the baths (Powell & Clark 2001), but the effect was variable depending on water quality and toxicity occurred at 50 p.p.m. for each chemical. Whether or not these treatments lead to a greater cure rate of the disease has not yet been determined.

Control

To date control of AGD of salmonids in Tasmania has revolved mainly around the timing of freshwater baths, some of which are regarded as prophylactic but are more likely to be counter-productive (Douglas-Helders, Nowak & Carson 2001). Some farmers avoid the problem entirely or partially by utilizing low salinity sites for all or part of the marine culture phase.

To date the Tasmanian industry has kept multiple classes of stock (spring smolts, out-of-season smolts, etc.) on a single site and, although no objective data are available, the apparent increase in virulence of *N. pemaquidensis* with serial passage under experimental conditions suggests that all-in all-out strategies may be more desirable. Similarly, increased biomass at individual lease sites inevitably reduces distances between cages and thereby makes transfer of infection from cages with clinically affected fish to other cages more likely. Undoubtedly, the effects of management strategies on the occurrence and severity of AGD will require more intensive investigation in the future.

Although chinook salmon are more difficult to culture than Atlantic salmon and rainbow trout there could be merit in having at least a proportion of these fish at a site if their apparent, relative resistance to AGD can be confirmed.

Conclusions

AGD of salmonids caused by *P. pemaquidensis* is a major constraint on salmonid mariculture in Tasmania and Spain. It is also a cause of intermittent, serious disease in Atlantic salmon in Ireland, France and North America. The economic impact of AGD on the turbot industry in southern Europe is not well-documented but the potential for significant morbidity and mortality is apparent.

For salmonids, treatment by freshwater baths is the only proven therapy but is stressful on the fish and for some operators can impose substantial managerial and financial imposts. In view of the relatively low salinity at which AGD occurs in turbot it is doubtful if freshwater bathing will prove to be as successful as with salmonids.

Research to date has not shown that immunization is likely to be an useful prophylactic measure although the use of extracellular products (ECP) as antigens has not been explored.

There is a great lack of information relating to the effects of such managerial practices as keeping a number of classes of fish at one site and increasing overall stocking levels at individual sites.

If control is to be achieved then a number of areas of research need to be investigated. These include:

1. Resistance and immune factors
   (i) It is anomalous that very little is known about the effects, if any, of a freshwater bath on the immune status of salmonids with AGD. Studies to elucidate this may well provide information which will enable better use of immunomodulators.
   (ii) Research on possible immune responses to ECP could lead to development of protective vaccines – something which has been elusive to date.
   (iii) Identification of lectin/receptor complexes responsible for *N. pemaquidensis* colonizing the gills could lead to the production of vaccines targeting key lectins involved in adherence.
   (iv) As has been accomplished for other diseases there could be selection for resistance to AGD. The present system of using ever-increasing numbers of freshwater baths ensures the continued susceptibility of the salmonid stocks.
   (v) The apparent, inherent resistance of chinook salmon to AGD needs to be scientifically tested.
2. Parasite factors
   (i) More research is required to identify differences (apart from pathogenicity) between 'wild'

and cultured *N. pemaquidensis* especially as such crucial procedures such as serology and PCR are based on cultured organism.

(ii) More studies are required to understand salinity preferences of various strains of *N. pemaquidensis*. Indeed, it is essential that historic and contemporary isolates are compared because it is possible that amoebae regularly exposed to freshwater may become tolerant of low salinities.

3. Management factors

(i) The effects of introducing sequential populations of naïve fish onto a single production site needs to be monitored.

(ii) Epidemiological modelling could be used to forecast the potential effects of increasing fish and/or cage numbers at any one site. In addition to such factors as increased exposure of uninfected/lightly infected cages to *N. pemaquidensis*, other complications such as the increased difficulty of adequately treating all fish under the changed conditions could be factored into any model.

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